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**(54) Title:** RECOMBINANT AGENTS AFFECTING THROMBOSIS

**(57) Abstract**

Analogues of Factor Xa (Factor Xai) which are inactive as proteases in the prothrombinase reaction are useful in treatment of diseases characterized by thrombosis. These antithrombotic agents can be conveniently prepared using recombinant techniques. In addition, modified forms of Factor Xa that generate Factor Xa in serum but have extended half-lives are useful in treating hemophilic conditions. These modified forms of Factor Xa are typically acylated forms of the serine residue in the active site which are slowly deacylated *in vivo*.

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-1-

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RECOMBINANT AGENTS AFFECTING THROMBOSIS

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Technical Field

The invention relates to peptide drugs for prevention or treatment of thrombosis. More specifically, the invention concerns analogs of Factor Xa which lack protease activity and which interfere with the ability of endogenous Factor Xa to effect the conversion of prothrombin to thrombin. In addition, the invention provides stabilized forms of Factor Xa for the treatment of hemophilia.

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Background Art

Thrombin is a multifunctional protease that regulates several key biological processes. For example, thrombin is among the most potent of the known platelet activators. In addition, thrombin is essential for the cleavage of fibrinogen to fibrin to initiate clot formation. These two elements are involved in normal hemostasis but in atherosclerotic arteries can initiate the formation of a thrombus, a major factor in pathogenesis of vasoocclusive conditions such as myocardial infarction, unstable angina, nonhemorrhagic stroke and reocclusion of coronary arteries after angioplasty or thrombolytic therapy. Thrombin is also a potent inducer of smooth cell proliferation and may therefore be involved in a variety of proliferative

-2-

responses such as restenosis after angioplasty and graft-induced atherosclerosis. In addition, thrombin is chemotactic for leukocytes and may therefore play a role in inflammation. (Hoover, R.J., et al. Cell (1978)

5 14:423; Etingin, O.R., et al., Cell (1990) 61:657.)

These observations indicate that inhibition of thrombin formation or inhibition of thrombin itself may be effective in preventing or treating thrombosis, limiting restenosis and controlling inflammation.

10           The formation of thrombin is the result of the proteolytic cleavage of its precursor prothrombin at the Arg-Thr linkage at positions 271-272 and the Arg-Ile linkage at positions 320-321. This activation is catalyzed by the prothrombinase complex, which is  
15 assembled on the membrane surfaces of platelets, monocytes, and endothelial cells. The complex consists of Factor Xa (a serine protease), Factor Va (a cofactor), calcium ions and the acidic phospholipid surface. Factor Xa is the activated form of its precursor,  
20 Factor X, which is secreted by the liver as a 58 kd precursor and is converted to the active form, Factor Xa, in both the extrinsic and intrinsic blood coagulation pathways. It is known that the circulating levels of Factor X, and of the precursor of Factor Va, Factor V,  
25 are on the order of  $10^{-7}$  M. There has been no determination of the levels of the corresponding active Factors Va and Xa.

          The complete amino acid sequences of human Factor X and Factor Xa are known. Figure 1 shows the  
30 complete sequence of the precursor form of Factor X as described by Davie, E.W., in Hemostasis and Thrombosis, Second Edition, R.W. Coleman et al. eds. (1987) p. 250. Factor X is a member of the calcium ion binding, gamma carboxyglutamyl (Gla)-containing, vitamin K dependent,  
35 blood coagulation glycoprotein family, which also

-3-

includes Factors VII and IX, prothrombin, protein C and protein S (Furie, B., et al., Cell (1988) 53:505).

As shown in Figure 1, the mature Factor X protein is preceded by a 40-residue pre-pro leader sequence which is removed during intracellular processing and secretion. The mature Factor X precursor of Factor Xa is then cleaved to the two-chain form by deletion of the three amino acids RKR shown between the light chain C-terminus and activation peptide/heavy chain N-terminus. Finally, the two chain Factor X is converted to Factor Xa by deletion of the "activation peptide" sequence shown at the upper right-hand portion of the figure (numbered 1-52), generating a light chain shown as residues 1-139, and a heavy chain shown as residues 1-254. These are linked through a single disulfide bond between position 128 of the light chain and position 108 of the heavy chain. As further indicated in the figure, the light chain contains the Gla domain and a growth factor domain; the protease activity resides in the heavy chain and involves the histidine at position 42, the aspartic at position 88, and a serine at position 185, circled in the figure.

There are two known pathways for the activation of the two-chain Factor X in vivo. Activation must occur before the protease is incorporated into the prothrombinase complex (Steinberg, M., et al., in Hemostasis and Thrombosis, Coleman, R.W., et al. eds. (1987) J.B. Lippencott, Philadelphia, PA, p. 112). In the intrinsic pathway, Factor X is cleaved to release the 52-amino acid activation peptide by the "tenase" complex which consists of Factor IXa, Factor VIII and calcium ions assembled on cell surfaces. In the extrinsic pathway, the cleavage is catalyzed by Factor VIIa which is bound to a tissue factor on membranes. Of interest herein, however, is the ability to convert Factor X to

-4-

Factor Xa by in vitro cleavage using a protease such as that contained in Russel's viper venom. This protease is described by DiScipio, R.G., et al., Biochemistry (1977) 6:5253.

5                   In some of the agents of the present invention, it is desirable to interfere with the functioning of Factor Xa in order to prevent excessive clotting. However, in hemophiliacs, the converse problem would be assisted by providing a source of Factor Xa independent  
10 of the activation process that takes place in normal individuals. Both of the common forms of hemophilia involve deficiencies in only the intrinsic pathway of activation, but the operation of the extrinsic pathway does not appear to be successful in arresting bleeding.

15                   The most common forms of hemophilia are hemophilia A which reflects a deficiency in the functioning of Factor VIII and hemophilia B which reflects a deficiency in the functioning of Factor IX (also known as Christmas factor). These forms of  
20 hemophilia are described by Levine, P.H., in "Plasma Coagulation Factors" (19\_\_), \_\_\_\_\_ ed., \_\_\_\_\_ Publishing Co., pp. 97-110. Since a deficiency in either of these factors results in an inadequate supply of Factor Xa, provision of Factor Xa  
25 should be effective in treatment of both hemophilias. In addition, a number of instances have been found wherein Factor X itself is incapable of providing an active Factor Xa. This relatively rare class of congenital disorders has been described, for example, by Reddy,  
30 S.B., et al., Blood (1989) 74:1486-1490; Watzke, H.H., et al. J Biol Chem (1990) 285:11982-11989; Hassan, H.J., et al., Blood (1988) 71:1353-1356; Fair, D.S., et al., Blood (1989) 73:2108-2116; and by Bernardi, F., et al., Blood (1989) 73:2123-2127.

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-5-

Factor Xa has not heretofore been useful as a pharmaceutical because of its extremely short half-life in serum which is only about 30 seconds. In the invention described below, the half-life of this medicament in serum is extended by providing a slow release acylated form wherein an acyl group bound to the serine at the active site inhibits clearance and is only slowly hydrolyzed to generate the active form of Factor Xa.

The use of acylation to prolong the half-life of blood clotting factors has been disclosed. For example, Cassels, R. et al. Biochem J (1987) 247:359-400 found that various acylating agents remained bound to urokinase, tPA and streptokinase-plasminogen activator complex for time periods ranging from a half-life of 40 minutes to a half-life of over 1,000 minutes depending on the nature of the acylating group and the nature of the factor. Acylation of tPA or streptokinase is also disclosed in U.S. Patent No. 4,337,244. The use of an amidinophenyl group functioning as an arginine analog to introduce, temporarily, a substituted benzoyl group into the active site for the purpose of enhancing serum stability was discussed by Fears, R. et al., Seminars in Thrombosis and Hemeostasis (1989) 15:129-139. This more general review followed a short report by Fears, R. et al. in Drugs (1987) 33: Supp. 3 57-63. Sturzebecher, J. et al. also reported stabilized acyl derivatives of tPA in Thrombosis Res (1987) 47:699-703. An additional report of the use of the acylated plasminogen streptokinase activator complex (APSAC) was published by Crabbe, S.J. et al. Pharmaco-therapy (1990) 10:115-126.

Returning to the function of Factor Xa per se, the activity of Factor Xa in effecting the conversion of prothrombin to thrombin is dependent on its inclusion in

-6-

the prothrombinase complex. The formation of the prothrombinase complex (which is 278,000 fold faster in effecting the conversion of prothrombin to thrombin than Factor Xa in soluble form) has been studied (Nesheim, H.E., et al., J Biol Chem (1979) 254:10952). These studies have utilized the active site-specific inhibitor, dansyl glutamyl glycyl arginyl (DEGR) chloromethyl ketone, which covalently attaches a fluorescent reporter group into Factor Xa. Factor Xa treated with this inhibitor lacks protease activity, but is incorporated into the prothrombinase complex with an identical stoichiometry to that of Factor Xa and has a dissociation constant of  $2.7 \times 10^{-6}$  M (Nesheim, M.E., J Biol Chem (1981) 256:6537-6540; Skogen, W.F., et al., J Biol Chem (1984) 256:2306-2310; Krishnaswamy, S., et al., J Biol Chem (1988) 263:3823-3824; Husten, E.J., et al., J Biol Chem (1987) 262:12953-12961).

Known methods to inhibit the formation of the prothrombinase complex include treatment with heparin and heparinlike compounds. This results in inhibition of the formation of the complex by antithrombin III in association with the heparin. Other novel forms of Factor Xa inhibition include lipoprotein-associated coagulation inhibitor (LACI) (Girard, T.J., et al., Nature (1989) 338:518; Girard, T.J., et al., Science (1990) 248:1421), leech-derived antistatin (Donwiddie, C., et al., J Biol Chem (1989) 264:16694), and tick-derived TAP (Waxman, L., et al., Science (1990) 248:593). Alternatively, agents which inhibit the vitamin K-dependent Gla conversion enzyme, such as coumarin, have been used. None of these approaches have proved satisfactory due to lack of specificity, the large dosage required, toxic side effects, and the long delay in effectiveness.

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-7-

Accordingly, the invention offers an alternative approach of enhanced specificity and longer duration of action in inhibiting the formation of an active prothrombinase complex.

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#### Disclosure of the Invention

The invention provides effective therapeutic agents for the prevention and treatment of thrombus formation and other pathological processes in the vasculature induced by thrombin such as restenosis and inflammation. This is highly significant as thrombus formation is the leading cause of death in Western societies, and restenosis is an expanding problem with increased use of angioplasty and other invasive procedures. The therapeutic materials of the invention are inactive forms of human Factor Xa which are nevertheless capable of incorporation into the prothrombinase complex, thus preventing the formation of active prothrombinase complex from endogenous Factor Xa. These pharmaceuticals are especially useful in acute settings to prevent thrombosis. This includes preventing thrombus formation in the coronary arteries of patients with rest angina, preventing rethrombosis after thrombolysis, and prevention of thrombosis during complicated angioplasties. These pharmaceuticals will also be useful in preventing smooth muscle cell proliferation following angioplasty or other vascular invasive procedures. The invention therapeutics offer considerable advantage over the now standard treatment which involves heparin (Hanson, R.S., et al., Proc Natl Acad Sci (1988) 85:3184). The compounds of the invention are double- or single-chain polypeptides which are capable of participation in the prothrombinase complex, but which result in an inactive complex.

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-8-

In one aspect, the invention is directed to a two-chain polypeptide, designated Factor Xai, which is capable of forming the prothrombinase complex, but which results in a complex that lacks proteolytic activity.

5 This two-chain polypeptide may be formed from one of two types of novel precursors. One type, designated herein Factor Xi, has substantially the amino acid sequence of Factor X, but is modified as described herein so as to result in an inactive two-chain polypeptide, Factor Xai,  
10 when cleaved by normal coagulation processing proteases or by in vitro treatment with Factor X activator from viper venom. The other type, designated herein Factor X'i, is a truncated form of single chain Factor X wherein the proteolytic cleavage site (or portion or extension  
15 thereof) at the C-terminus of the light chain, shown as RKR in Figure 1, is ligated directly (with the optional addition of one or several amino residues) to the N-terminus of the activated form of the heavy chain as shown in one embodiment in Figure 3. Upon cleavage,  
20 Factor X'i also results in the two-chain Factor Xai of the invention which results in a prothrombinase complex lacking proteolytic activity. Of course, the active cofactor, Factor Xa, could also be generated by using the analogous precursors of the Factor X' type illustrated in  
25 Figure 2.

Thus, in other aspects, the invention is directed to the Factor Xai two-chain prothrombinase complex, and to the novel precursors of the Factor Xai therapeutic proteins, to the DNA sequences encoding them,  
30 and to recombinant materials and methods generally which permit their production.

Other aspects of the invention include pharmaceutical compositions of the therapeutically useful Factor Xai proteins and to methods to prevent or treat

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-9-

thrombosis or other pathological events initiated by thrombin using these compositions.

The availability of recombinantly produced Factor Xa provides a convenient source for this material for use in the treatment of hemophilia. The factor, whether recombinantly produced directly, obtained from recombinantly produced Factor X by activation using, for example, Russell's Viper Venom, or isolated from plasma and similarly converted to Factor Xa can be converted to form a usable pharmaceutical by extending its half-life in serum. This can be accomplished by acylation of the serine at the active site which provides a slow release form of the active factor.

Thus, in an additional aspect, the invention is directed to Factor Xa wherein the serine residue at position 185 of the heavy chain is acylated with an agent which permits its appropriately timed conversion to active Factor Xa. Other aspects of the invention include pharmaceutical compositions for the treatment of hemophilia containing the acylated Factor Xa of the invention and to methods to treat hemophilia using these compositions.

#### Brief Description of the Drawings

Figure 1 shows the structure of human Factor X and its relevant cleavage sites as described in the prior art.

Figure 2 shows the structure of one embodiment of a single-chain Factor X' which is a precursor to yield a two-chain cleavage product that will participate in prothrombinase complex formation. The form shown in this figure will produce a two-chain peptide which retains proteolytic activity in the complex; a modified form, as described below, is catalytically inactive.

Figure 3 shows one embodiment of Factor X'i.

-10-

Figure 4 shows the cDNA sequence encoding Factor X.

Figure 5 is a Western blot of recombinantly produced, potentially active Factor X and Factor Xa.

5 Figure 6 is a Western blot of recombinantly produced, inactivated forms of Factor X and Factor Xa.

Figures 7a-7d consist of a series of Lineweaver-Burk plots showing the enzymatic activity of native and recombinantly produced Factor X converted to  
10 activated form.

Figures 8a-8d show a comparison of prothrombinase complex activity of various Factor X forms.

Figure 9 shows the result of a two-stage  
15 prothrombin clotting assay for various forms of Factor X.

Figure 10 shows inhibition of prothrombinase complex formation by inactive forms of Factor X.

#### Modes of Carrying Out the Invention

20 In general, one aspect of the invention encompasses the therapeutically useful two-chain polypeptide, designated Factor Xai herein, and the single-chain precursors of this two-chain protein. These peptides are about 80% homologous, preferably about 90%  
25 homologous to the amino acid sequences shown at positions 1-139 (light chain) and 1-254 (heavy chain) in Figure 1. It should be noted that in Figure 1, the pre-pro leader sequence is numbered -40 through -1, prior to the numbering beginning at the N-terminus of the light chain.  
30 The light chain is numbered 1-139. The intervening tripeptide RKR, which, in mature Factor X, is deleted, is not numbered. The activation peptide beginning subsequent to this intervening tripeptide is numbered 1-52; the isoleucine referred to hereinbelow as "position  
35 53" of the activation peptide is, in fact, the first

-11-

amino acid of the heavy chain in the activated form. This restarts the numbering shown in the figure, and the heavy chain is numbered 1-254.

The embodiments of the two-chain peptide, Factor Xai, are effective in forming the prothrombinase complex, as determined by their ability to inhibit (or compete with) the formation of the native prothrombinase complex involving Factor Xa. Their ability to inhibit prothrombinase complex formation can be determined conveniently by the method of Krishnaswamy, S., J Biol Chem (1988) 263:3823-3834, cited above. However, when incorporated into the prothrombinase complex, the complex fails to show its proteolytic activity, as determined by the method of van Dieijen, G., et al., J Biol Chem (1981) 256:3433 or of Skogen, W.F., et al., J Biol Chem (1984) 256:2306. These Factor Xai proteins may or may not be immunoreactive with antibodies raised against native Factor Xa or against Factor X, including commercially available antibodies specific for human Factor X. The Factor Xai proteins are antithrombotic materials.

The invention is also directed to precursors of the foregoing inactive competitors with Factor Xa. One group of these precursors are novel modified forms of Factor X designated Factor X', wherein one or more of the residues at position 42, 88 or 185 of the heavy chain are converted to alternate amino acid residues, thus inactivating the proteolytic properties of the peptide. The modified forms of Factor X contain at a minimum the light chain sequence and the heavy chain sequence to which is attached the activation peptide. The intervening tripeptide (between the C-terminus of the light chain the N-terminus of the activation peptide) and the pre-pro leader sequence may or may not be present. Thus, the Factor X may either be a single-chain protein (when the tripeptide is included) or a two-chain

-12-

precursor of Factor Xa (when the tripeptide has been deleted).

Preferably, the alteration at the residues of the protease active site is either a deletion, or a conversion to a conservative, substituted amino acid so as to maintain the three-dimensional conformation of the two-chain protein. By "conservative" is meant a substitution which maintains the correct conformation, rather than a substitution which maintains the correct activity. Thus, the histidine residue at position 42 is preferably replaced by phenylalanine; the aspartic acid at position 88 is preferably replaced by asparagine or glutamine, and the serine residue at position 185 is preferably replaced by alanine or glycine.

Another group of precursors to the antithrombotic dimeric peptides of the invention is designated Factor X'i. In Factor X'i precursors, at least a substantial portion of the activation peptide, preferably the entire activation peptide, is deleted. The precursors to the two-chain form of Factor X'i, however, must retain a proteolytic cleavage site between the light and heavy chains. Therefore, amino acids subject to endogenous proteolysis are conveniently included in a single-chain precursor form which extends the carboxy terminus of the light chain by virtue of the cleavage site to the N-terminus of the heavy chain. A typical embodiment of the single-chain precursor (including the pre-pro leader) to the two-chain Factor X'i, which will now automatically be activated by virtue of the absence of the activation peptide sequence (thus, becoming a Factor Xai) is shown in Figure 3. In this embodiment, the hexapeptide sequence RKRRKR connects the C-terminus of the light chain directly to the isoleucine residue at the N-terminus of the heavy chain. Cleavage of this single-chain Factor X'i results in X'ai.

-13-

In constructing such modified X'i type precursors, a hydrophobic amino acid must be retained at the N-terminus of the heavy chain (natively isoleucine). See, for example, Dayhoff, M.O., "Atlas of Protein Sequence and Structure" (1972) 5:\_\_\_\_\_ (Biomed. Res. Foundation, Wash. D.C.) and Greer, J., J. Molec. Biol. (1981) 153:1043-1053.

It is evident that the single-chain Factor X' precursors are also novel and, when cleaved by proteolysis, yield Factor Xa, the normal enzymatically-active form of the dimeric protein. This corresponding construction is shown in Figure 2.

Thus, when the single-chain precursors of either Factor Xa or Factor Xai are produced recombinantly in suitable host cells, the endogenous enzymes of the host cell may (1) cleave the single-chain precursor Factor X or X' to a two-chain form and, in the case of single-chain Factor X or X', further activate the factor by cleavage of the activation peptide; in the case of Factor X' single-chain precursors, there is no activation peptide present, so the single-chain precursor will automatically be activated when cleaved into a dimeric peptide. For Factor X precursors, the double-chain form containing the activation peptide may also be cleaved in vitro using a suitable protease, such as the Factor X activator of Russell's viper venom. Either Factor Xa or Factor Xai will be obtained depending on whether the active site has been inactivated by alteration at the appropriate codons as further described hereinbelow.

To summarize the terminology used in this application, the following glossary may be useful:

"Factor X" refers to the native or recombinantly produced single- or two-chain Factor X sequence, essentially as shown in Figure 1, containing at a minimum the heavy chain to which is attached the

-14-

activation peptide, at its N-terminus, and the light chain. These may or may not be linked through a cleavage sequence as indicated in the figure. "rX" refers specifically to the recombinantly produced form of this factor.

"Factor Xi" refers to the recombinantly produced form of Factor X which lacks proteolytic activity by virtue of the modification of the active site as described above. The designation "rXi" is also used for this protein.

"Factor Xa" refers to native or recombinantly produced, enzymatically active dimer containing light and heavy chain only. The activation peptide is not present in this complex. "rXa" refers specifically to this complex when produced recombinantly.

"Factor Xai" refers to the modified form of Factor Xa which is activated in the sense that it combines to form the prothrombinase complex, but which has no serine protease activity by virtue of the modification of its active site. As this protein is produced only by recombinant methods, "rXai" is also used to designate this complex.

"Factor X'" refers to a modified, single-chain form of Factor X which includes only the light chain, heavy chain, and an intermediate, specific proteolytic cleavage site, such as that shown in Figure 2. This single-chain precursor may also contain the pre-pro sequence. As it is a result only of recombinant production, it is also designated "rX'." When cleaved by a protease so as to become activated, the products are indistinguishable from Factor Xa (or rXa) and, accordingly, this terminology is again used.

Similarly, "Factor X'i" refers to a modified form of Factor X' which has been inactivated at its catalytic site as described above. One form is shown in



-15-

Figure 3. Upon conversion to the two-chain form, as the activation peptide is not present in the precursor, the products are indistinguishable from Factor Xai or rXai.

"Acylated Factor Xa" or "AcXa", unless  
5 otherwise specified, refers to Factor Xa, whether produced recombinantly or not, wherein the serine residue at position 185 has been blocked with a substituent which provides the Factor Xa with a half-life in serum of at least 5-10 minutes, preferably more than 15 minutes, and  
10 which releases Factor Xa in active form over this time period. The half-life in serum can be measured directly in vivo using a suitably labeled form. However, it is preferable to assess the ability of the extended life AcXa to generate the active factor within the required  
15 time frame in vitro using as a criterion in vitro assays for which Xa is a catalyst. Under these conditions, suitable forms of AcXa for the invention include those which have a rate constant for hydrolysis in isotonic aqueous media at pH 7.4 and 37°C such that a half-life of  
20 approximately 5 minutes-several hours is achieved. The half-life can be determined directly in vitro by measuring the rate of hydrolysis of the acylated Xa, if desired, using its ability to activate clotting, or the prothrombinase reaction as criteria for Xa formation.

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#### Preparation of the Invention Peptides

The genomic organization and coding sequence for human Factor X are known and the cDNA has been retrieved and sequenced (Leytus, S.P., et al., Proc Natl Acad Sci USA (1984) 81:3699; Kaul, R.K., et al., Gene (1986) 41:311-314). The complete cDNA sequence is shown in Figure 4.

Full-length Factor X cDNA inserts are subcloned into M13mp18 or M13mp19 vectors for site-directed  
35 mutagenesis. (The correct sequence encoding Factor X is

-16-

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verified by dideoxy sequencing.) Standard modification techniques are now readily available in the art, and thus the sequence encoding Factor X is modified to obtain the DNA-encoding Factor X', Factor Xi, and Factor X'i.

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The modified coding sequences for Factor X', Factor Xi and Factor X'i are then ligated into suitable expression vectors for recombinant production of the polypeptides. In the expression vectors, the prepro leader sequence is preferably retained for expression in compatible host cells such as mammalian hosts. If bacterial or yeast expression is desired, it may be desirable to substitute a compatible leader sequence, such as the penicillinase sequence in bacteria, or the alpha-factor sequence in yeast. Alternatively, an ATG start codon may be directly placed before amino acid 1 of the light chain-encoding sequence to produce an intracellular protein.

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The choice of host and expression control system is governed by the nature of the desired result. If endogenous activation by proteolytic cleavage is desired, mammalian systems may be preferable. However, production in microorganisms which provide simplicity of culturing is not precluded, provided an in vitro system for carboxylation to produce the required carboxyglutamyl residue is employed, or the microorganism or other host natively lacking this posttranslational processing system is transformed to provide it. A wide variety of expression systems for recombinant DNA sequences is known in the art.

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-16a-

The modified DNA encoding Factor X', Factor Xi or Factor X'i is preferably provided with linkers for ligation into cloning and expression vectors. Techniques for preparation of such vectors are well understood in the art. The DNA encoding the desired Factor X', Factor Xi or Factor X'i is ligated in operable linkage with control sequences, including promoters, upstream enhancers, termination sequences, and so forth, depending on the nature of the intended recombinant host cells.

10 Technology is currently available for expression of

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-17-

heterologous genes in a variety of hosts, including procaryotic hosts and various eucaryotes, including yeasts, mammalian or avian or insect cells, and plant cells. The choice of control sequences and markers in the expression vectors is selected appropriately to these hosts.

For example, in procaryotic hosts, various promoters, including inducible promoters such as the trp promoter and lambda phage  $P_L$  promoter can be employed. Hybrid promoters such as the tac promoter, which contains the trp polymerase binding region in combination with the lac operator, can be used. Suitable markers are generally those related to antibiotic resistance. On the other hand, in mammalian cell cultures, commonly used promoters are virally derived, such as the early and late SV40 promoters and adenovirus promoters. Mammalian regulatable promoters, such as the metallothionein-II promoter may also be used. The metallothionein-II promoter is regulated by glucocorticoids or heavy metals. These promoter systems are compatible with typical mammalian hosts, the most commonly used of which is Chinese hamster ovary (CHO) cells.

Another commonly employed system is the baculovirus expression system compatible with insect cells. Plant cells, used in conjunction with, for example, the nopaline synthetase promoter, and yeast cells, used in conjunction with promoters associated with enzymes important in the glycolytic pathway, can also be employed. A number of suitable expression systems can be found in appropriate chapters in "Current Protocols in Molecular Biology," Ausubel, F.M., et al., eds., published by Wiley Interscience, latest edition.

The AcXa of the invention is prepared by standard acylation reaction of Factor Xa, whether recombinantly produced or isolated from plasma, according

-18-

to procedures analogous to those set forth, for example, or referenced in Cassels, R. et al., Biochem J (1987) 247:395-400 or U.S. Patent No. 4,337,244 cited above. Suitable esters include the 4-toluoyl ester, the 3,3-  
5 dimethyl acrylyl ester, cyclohexylideneacetyl ester, the cyclohex-1-enecarbonyl ester, the 1-methylcyclohexylideneacetyl ester, the 4-aminobenzoyl ester, the r-guanidinobenzoyl ester, the 4-anisoyl ester, the 4-N,N-dimethylaminobenzoyl ester, and the PDAEB (4-N-(2-N'(3-  
10 (2-pyridyldithio)-propenyl)amino-ethyl)aminobenzoyl ester. In general, the acylating agent will be the activated form of a non-toxic acid which provides a saturated, unsaturated or aromatic 5- or 6-carbon ring to which a carboxyl is substituted. The ring may contain  
15 further substitutions, such as amino, alkoxy, alkyl, additional ring systems, or any other non-interfering non-toxic substituent. Any compound capable of acylating the serine hydroxyl group or otherwise blocking serine in a reversible manner is suitable for synthesis of the  
20 AcXa. As described in U.S. Patent No. 4,337,244 in general, either direct or inverse acylating agents can be used. For direct acylating agents, the acylating moiety is itself attracted to the catalytic site of Factor Sa; in the inverse acylating approach, the leaving group is  
25 thus attracted. The acylated form of Xa is then purified from the reaction mixture using standard purification techniques, including dialysis, chromatography, selective extraction, and the like.

The compounds of the invention which serve as  
30 AcXa pharmaceuticals must have an appropriate deacylation rate which assures an appropriate clearance time in vivo. The deacylation rate can be measured as having a half life of at least 5 minutes in vitro in buffer using prothrombinase and/or clotting assays. Deacylation can  
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-18a-

be measured directly as described by Smith, R.A.G., et  
al., "Progress in

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-19-

Fibronolosis" (1985) Vol. VII, pp. 227-231 (Churchill Livingstone). Prothrombinase and clotting assays are described by Wolf, D.L., et al. J Biol Chem (1991) (in press).

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#### Administration and Use

The Factor Xai peptides of the invention are prothrombinase inhibitors and are thus useful in procedures complicated by thrombosis and in conditions whose pathogenesis involves thrombin generation. These conditions include those involving arterial thrombosis, such as unstable (i.e., rest) angina and abrupt vessel closure during vascular interventions including coronary and peripheral angioplasty and atherectomy, and during and after vascular bypass procedures (peripheral and coronary), reocclusion after thrombolytic therapy for myocardial infarction, thrombotic stroke (stroke in evolution), and thrombosis due to vasculitis (Kawasaki's disease). Also included are conditions involving venous thrombosis, such as deep venous thrombosis of the lower extremities, pulmonary embolism, renal vein, hepatic vein, inferior vena cava thrombosis, and cavernous sinus thrombosis. Other target conditions are those involving diffuse activation of the coagulation system, such as sepsis with disseminated intravascular coagulation, disseminated intravascular coagulation in other settings, thrombotic thrombocytopenic purpura, and rare conditions of unknown etiology (Lupus anticoagulant).

The Factor Xai of the invention is also useful as an anticoagulant and anti-inflammatory for cardiopulmonary bypass, in harvesting organs, in preparation of blood products or samples and in transport and implantation of organs and associated treatment of the recipient. The Factor Xai, in a slow release form,

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-20-

is especially useful in indwelling intravascular devices (i.v.s, catheters, grafts, patches).

Thrombosis also plays a role in restenosis following vascular interventions such as angioplasty, atherectomy, or endarterectomy by directly or indirectly causing smooth muscle cell proliferation, and the Factor Xai of the invention is also useful in treating this condition.

Adult respiratory distress syndrome (ARDS) is thought to be an "endotoxin" disease in which a prothrombotic endothelium is likely to exist, with inflammatory and proliferative components; Factor Xai is also useful in treatment of ARDS.

The therapeutic Factor Xai peptides of the invention are formulated for administration using excipients conventional for administration of proteins, typically by injection, as set forth, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Company, latest edition, Easton, PA. For the antithrombosis effect, the Factor Xai proteins are administered systemically, preferably by injection, and preferably by intravenous injection. Dosage levels depend on a number of factors, including the condition of the subject and the specific Factor Xai embodiment chosen. However, suitable dosage ranges are on the order of 1-50 mg per patient per continuous injected dose. For injection, the protein is dissolved or suspended in liquid medium, for example, Hank's solution, Ringer's solution, dextrose solution, and various buffers. Additional excipients such as stabilizers can also be employed.

Besides injection, the peptides of the invention can be administered systemically, via suppository, oral administration, transmucosal administration, including intranasal sprays, and by slow



-21-

release formulations. Additional formulation techniques include encapsulation formulations, such as liposomes.

In addition to utility as a therapeutic, the Factor Xai can be used to raise polyclonal antisera or to  
5 produce cells which can be fused to immortalizing partners to obtain sources of monoclonal antibodies specific for this peptide. These antibodies are useful as passive therapeutics or as diagnostic tools.

The Factor Xa of the invention which has been  
10 modified to extend its half-life in vivo is useful in treating hemophilia whether the origin of the hemophilia resides in the Factor X gene, or the more widespread types, hemophilias A and B. In this application, the extended half-life Factor Xa is administered parenterally  
15 or systemically using dosage levels and formulations similar to those set forth above for Factor Xai. Administration is typically by injection, but other systemic methods such as transmucosal, transdermal and the like can also be used. The formulations for these  
20 routes of administration are analogous to those set forth above. In particular, it is advantageous to utilize various vesicles or slow release systems as is generally known in the art. For example, Giles, A.R., et al. Brit J Hematol (1988) 69:491-497 describe the formulation of  
25 Factor Xa in phosphatidylcholine-phosphatidylserine vesicles. Similarly the extended life Factor Xa may be administered in this fashion.

The following examples are intended to  
30 illustrate, but not limit the invention.

-22-

Example 1Construction of DNA Encoding Catalytically  
Inactive Forms of Recombinant Human Factor X (rXi)

A full length cDNA clone for human Factor X was  
5 obtained from Dr. W.R. Church, University of Vermont  
(Figure 4). This cDNA encodes the amino acid sequence of  
Figure 1 or an allelic variant. This human Factor X cDNA  
was cloned into EcoRI site of vector pBSII (Stratagene)  
to obtain pBSX. The HindIII-XbaI fragment of pBSX  
10 comprising the entire Factor X coding region was  
subcloned into the Hind III-XbaI site of vector M13mp19  
(Mp19X). Oligonucleotide site-directed mutagenesis was  
then performed as described by Kunkel, T.A., et al.,  
Methods in Enzymol (1987). 154:367.

15 The following forms were produced:

The oligomer TGC CGA GGG GAC GCC GGG GGC CCG  
CAC was used to convert serine (S<sub>185</sub>) at position 185aa  
on the Factor X heavy chain to alanine (A<sub>185</sub>) to obtain  
rXiA<sub>185</sub>.

20 The oligomer ACC TAT GAC TTC AAC ATC GCC GTG  
CTC was used to convert aspartic acid (D<sub>88</sub>) at position  
88aa on the Factor X heavy chain to asparagine (N<sub>88</sub>) to  
obtain the gene encoding rXiN<sub>88</sub>.

Both oligomers were used to obtain the gene  
25 encoding rXiN<sub>88</sub>A<sub>185</sub>. (See Figure 1 for location of these  
sites). Verification of oligonucleotide-directed  
mutagenesis was accomplished by dideoxy sequencing.

Example 2

30 Construction of DNA Encoding the  
Truncated Precursor of Human Factor Xa (rX')

The cDNA of human Factor X (Mp19X) was  
converted to encode various truncated forms of human  
Factor Xa, collectively designated rX', by deletion of  
35 the activation peptide, by oligonucleotide site directed

-23-

mutagenesis (Kunkel, T.A., et al., Methods in Enzymol (1987) 154:367). The following oligonucleotides employed with the corresponding amino acid changes are as follows:

5 rX'Δ0: ACC CTG GAA CGC AGG AAG AGG ATC GTG GGA  
GGC CAG GAA TGC, which aligned arginine (R<sub>142</sub>) following the C-terminus of the Factor X light chain with isoleucine (I<sub>53</sub>) 53aa of the Factor X activation peptide (1aa of the heavy chain);

10 rX'Δ1: ACC CTG GAA CGC AGG AAG AGG AGA ATC GTG  
GGA GGC CAG GAA TGC, which aligned this R<sub>142</sub> with arginine (R<sub>52</sub>) of the Factor X activation peptide;

rX'Δ2: ACC CTG GAA CGC AGG AAG AGG CGG AAA AGA  
15 ATC GTG GGA GGC CAG GAA TGC, which extended R<sub>142</sub> following the Factor X light chain by two amino acids arginine (R<sub>143</sub>) and lysine (K<sub>144</sub>) and aligned this  
terminus with R<sub>52</sub> of the Factor X activation peptide (Figure 2);

rX'Δ3: ACC CTG GAA CGC AGG AAG AGG CCT AGG CCA  
20 TCT CGG AAA CGC AGG ATC GTG GGA GGC CAG GAA TGC, which extended R<sub>142</sub> following the Factor X light chain by seven amino acids, Proline (P<sub>143</sub>) Arginine (R<sub>144</sub>) Proline (P<sub>145</sub>) Serine (S<sub>146</sub>) Arginine (R<sub>147</sub>) Lysine (K<sub>148</sub>) Arginine (R<sub>149</sub>) as described in Ehrlich, H.J., et al., J Biol Chem (1989) 264:14298, and aligned this terminus  
25 with R<sub>52</sub> of the Factor X activation peptides.

Verification of the oligonucleotide directed mutagenesis was accomplished by dideoxy sequencing.

As will be further described below, the precursor derived from rX'Δ2 was cleaved endogenously  
30 when recombinantly produced in CHO cells to obtain directly the activated form rXa. The precursor derived from rX'Δ0 was not cleaved endogenously in CHO cells when produced recombinantly. The precursor derived from rX'Δ1 or from rX'Δ3 was cleaved incompletely. The dimeric

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-24-

peptides derived from rX' $\Delta$ 0, rX' $\Delta$ 1 and rX' $\Delta$ 3 were not active enzymatically.

### Example 3

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#### Construction of DNA Encoding

#### Catalytically Inactive Truncated Precursor (rX'i)

cDNA Factor X' constructs described in Example 2 were converted to encode the catalytically inactive forms of X' (rX'i) by oligonucleotide site-directed  
10 mutagenesis as described in Example 1. These constructs included rX'i( $\Delta$ 2)<sub>N<sub>88</sub></sub> (rX'i( $\Delta$ 2)<sub>N<sub>88</sub></sub>) and rX'i( $\Delta$ 2)<sub>N<sub>88</sub></sub>A<sub>185</sub>, as shown in Figure 3.

### Example 4

15

#### Expression of the Genes Encoding

#### Precursor (rX and rX')

The expression vector pRC/CMV (Invitrogen) was modified by replacing the CMV promoter with the SR $\alpha$  promoter (Takabe, Y., et al., Molec Cell Biol (1988)  
20 8:466). The ClaI-XbaI fragment, filled in by Klenow polymerase at the ClaI site which contained the SR $\alpha$  promoter was isolated from the expression vector pBJ1 (Lin, A., et al., Science (1990) 249:677 and available from M. Davis, Stanford University) and subcloned into  
25 the NruI-XbaI site of pRC/CMV creating expression vector pBN. The StuI fragment of pBN, comprising the SR $\alpha$  promoter, bovine growth hormone polyadenylation site and M13 origin or replication was subcloned into the StuI site of pSV2DHFR generating expression vector pBD. The  
30 Mp19 SmaI-EcoRV fragments of the precursor DNAs described in examples were subcloned into the Klenow polymerase-filled-in XbaI site of pBN and pBD.

The resulting expression vectors were transfected into CHO by lipofection (BRL). Selection for  
35 transfected clones was by either 1 mg/ml G418 Neomycin

-25-

(Gibco) or 25 ng/ml Methotrexate (Sigma). Single clones were isolated by cloning cylinders, expanded and expression levels were determined on 24 hour serum free medium by a standard solid phase antibody capture assay (ELISA) as described by Harlow, E., and Lane, D., in Antibodies (1988), Cold Spring Harbor Laboratory, New York. The ELISA utilized a primary antibody of rabbit polyclonal antihuman Factor X (STAGO, American Diagnostics Inc.) and a rabbit-specific secondary antibody of peroxidase conjugated goat IgG.

Clones from constructs pBNX, pBNX' $\Delta$ O, pBNX' $\Delta$ 1, pBNX' $\Delta$ 2, and pBNX' $\Delta$ 3 were expanded to confluency in T-75 tissue culture flasks in RPMI medium supplemented with 10% fetal bovine serum, Penicillin, Streptomycin, Glutamine and 10  $\mu$ g/ml vitamin K, washed four times with serum free medium and incubated overnight with serum free medium.

Post-incubation the medium was harvested, centrifuged at 3000 rpm and 2 ml was precipitated with 10% Trichloroacetic acid (TCA). The TCA pellet was washed three times with 100% Acetone, resuspended to 0.05 ml SDS-PAGE sample buffer or 0.05 M SDS-PAGE sample buffer with 1M  $\beta$  Mercaptoethanol. Duplicate 10  $\mu$ l aliquots were electrophoresed on 12% SDS polyacrylamide gels and transferred to Immobilon filters (Millipore). Western blot analysis was performed with the primary human Factor X polyclonal rabbit sera (STAGO, American Diagnostics, Inc.) at a 1/4000 dilution in 1% nonfat dry milk, 0.1% NP40, 10 mM Tris-HCl pH 7.5, 150 mM NaCl. The secondary antibody was  $^{125}$ I labeled Fab donkey antirabbit IgG (Amersham). Autoradiography was overnight at -70°C with an intensifier screen.

The pattern of antibody reactivity showed that the expected products were produced. All five products, i.e., those derived from rX, rX' $\Delta$ O, rX' $\Delta$ 1, rX' $\Delta$ 2, and

-26-

rX' $\Delta$ 3 were positive in the above ELISA based on rabbit polyvalent human Factor X antisera. ELISAs were also performed with respect to mouse monoclonal antibodies Mab323, Mab743 and Mab325. Mab323 is specifically  
5 reactive with the activation peptide. Mab743 is reactive with either the activated or inactivated form of human Factor X. Mab325 is calcium ion dependent and directed to the light chain; this antibody reacts with the gamma-carboxylated region.

10 Supernatants from cultures containing any of the five constructs gave positive ELISAs with Mab743 and Mab325; thus, posttranslational GLA processing is indicated in all cases. All of the rX' mutants failed to react with Mab323 confirming the absence of the  
15 activation peptide.

Figure 5 shows Western blot analysis using polyclonal rabbit antisera of products derived from rX, rX' $\Delta$ 0, rX' $\Delta$ 1, rX' $\Delta$ 2, rX' $\Delta$ 3 and CHO control medium. Rabbit polyclonal antisera to X was not efficient in  
20 localizing the fully processed heavy chain of human Factor Xa; hence, in all cases the position expected to be occupied by the activated heavy chain does not appear. Figure 5a shows reduced and Figure 5b nonreduced forms of these recombinant proteins. Lane 1, 0.7  $\mu$ g native human  
25 Factor X (Dr. C. Esmon, OMRP, University of Oklahoma); Lane 2, rX; Lane 3, rX' $\Delta$ 0; Lane 4, rX' $\Delta$ 1; Lane 5, rX' $\Delta$ 2; Lane 6, rX' $\Delta$ 3; Lane 7, CHO control medium.

Figure 5a shows that the recombinant products of rX and rX' $\Delta$ 2 are dimeric proteins which are separable  
30 under reducing conditions. The products of expression of rX' $\Delta$ 0, rX' $\Delta$ 1 and rX' $\Delta$ 3 apparently are largely single-chain products. It appeared that the unprocessed Factor X' single chains comigrated anomalously with the heavy chain as shown in lanes 3-7, apparently due to the degree  
35 of proteolytic processing of the novel cleavage sites.

-27-

The failure of these X' precursor proteins to be processed properly was consistent with the results of a coagulation assay, described in Example 8, which demonstrated that Factor Xa, RVV-activated Factor X or recombinant Factor X and X' $\Delta$ 2 were comparably active, while the remaining X' secreted products were dramatically less efficient, by at least 5 orders of magnitude. The data with respect to enzyme activity are shown in Table 1:

Table 1

15	Factor X	RVV Activation	Catalytic Efficiency (%)	Coagu- lation
	X	+	100	+
	Xa	-	851	+
	rX	+	29.6	+
20	X' $\Delta$ 0	-	$5.2 \times 10^{-4}$	-
	X' $\Delta$ 1	-	$12.6 \times 10^{-4}$	-
	X' $\Delta$ 2	-	269	+
	X' $\Delta$ 3	-	$69.5 \times 10^{-4}$	-
	CHO	-	0	-

The column of Table 1 labeled "catalytic efficiency" shows the amidolytic substrate activities of the various factors, activated with RVV if necessary. The catalytic efficiencies shown are the ratio of  $k_{cat}/K_m$  and were normalized to the results for plasma Factor X. As shown in the table, both recombinant Factor X and X' $\Delta$ 2 were active in Factor X dependent 2PT clotting assays, while the enzymatic activities of the other recombinant proteins were 4 orders of magnitude lower.

-28-

From Figure 5b, it is apparent that the expression products of the X'-encoding gene are of lower molecular weight than rX or native Factor X.

5

Example 5Purification of rX and X' $\Delta$ 2

Both recombinant Factor X and X' $\Delta$ 2 were purified to homogeneity as follows: After growth to confluency, CHO cells transfected with pBNX or pBNX' $\Delta$ 2 were washed 4-5 times with serum-free media. The cells were then cultured for consecutive 24 hr periods at 37°C in serum-free media supplemented with 4  $\mu$ g/ml vitamin K<sub>3</sub>. Harvested media were centrifuged at 15,000 x g for 20 min followed by filtration of the supernatant through a 0.2  $\mu$ m filter. To the media was added Tris HCl, pH 7.5 to 20 mM, NaEDTA to 10 mM, and the resultant was chromatographed on Q-Sepharose Fast Flow (Pharmacia). All chromatographic steps were performed at 4°C. The columns were washed extensively with 20 mM Tris, pH 7.5, 10 mM EDTA, 0.15 M NaCl, and the proteins were eluted with 20 mM Tris, pH 7.5, 0.5M NaCl, 5 mM CaCl<sub>2</sub>. Peak fractions were pooled and either stored frozen at -20°C or applied directly to an anti-factor X monoclonal antibody affinity column as described by Church, W.R., et al., Throm Res (1985) 38:417-424. The antibody used for isolation (aHFX-1d, Mab B12-A3) is specific for human factor X, not influenced by Ca<sup>2+</sup>, and binds both factors X and Xa (unpublished data). Factor rX' was purified further on a benzamidine-Sepharose column (Pierce) as described by Krishnaswamy, et al., J Biol Chem (1987) 262:3291-3299. The concentrations of the proteins were determined by quantitative ELISA, colorimetric protein assay (Harlow, E., et al., "Antibodies, A Laboratory Manual" (1988), Cold Spring Harbor Laboratories, Cold Spring Harbor, New York), and by absorbance measurement



-29-

at 280 nm using extinction coefficient 11.6 and molecular weights of 58,900 for factor X, 46,000 for X' $\Delta$ 2.

The purified factors, when subjected to SDS-PAGE under reducing conditions and silver stained showed that the recombinantly produced Factor X was separated into 3 bands representing the full-length precursor (75 kD), the heavy chain containing the activation peptide (45 kD) and the light chain (22 kD). When amino terminal sequence analysis was performed following electrotransfer to nylon filters, the light chain was shown to be heterogeneous with 27% initiating at Val<sub>37</sub> and 73% initiating at Ala<sub>41</sub>; the 75 kD species was also heterogeneous with 41% initiating at Val<sub>37</sub> and 59% initiating at Ala<sub>41</sub>.

#### Example 6

##### Expression of Genes Encoding Inactivated Recombinant Human Factor X (rXi and rX'i)

The X' form chosen for conversion to the inactive form was the rX' $\Delta$ 2 form shown in Figure 4. pBN-derived cell lines for rX, rX'( $\Delta$ 2), rXiN<sub>88</sub>A<sub>185</sub>, rXiA<sub>185</sub>, rX'i( $\Delta$ 2)N<sub>88</sub>A<sub>185</sub> and rX'iN<sub>88</sub>( $\Delta$ 2) were grown to confluency in 800 cm<sup>2</sup> roller bottles as described in Example 4, washed four times with serum free medium and incubated overnight with 50 ml serum-free medium. The medium was replenished and harvested daily.

Consecutive harvests were pooled, centrifuged at 3000 rpm and passed directly through a Factor X-specific monoclonal antibody (Mab) affinity column (Mab717) supplied by Dr. C. Esmon (OMRF, University of Oklahoma). The bound "Factor X" was eluted from the Mab717 column with 80% ethylene glycol, dialyzed against 10 mM Tris HCl, pH 7.5, 150 mM NaCl and concentrated on a Centricon 10 filtration unit (Amicon). "Factor X" protein concentrations were determined by ELISA as

-30-

described in Example 4 utilizing serial dilution with comparison to a standard preparation of human Factor X (Haematologic Technologies, Inc., C. Esmon, OMRF, University of Oklahoma).

5           The purified proteins were characterized by Western blot analysis as outlined in Example 4. Figure 6 shows a Western blot of these  $\beta$ -mercaptoethanol-reduced, Mab717 purified recombinant human Factor X analogs. Lane 1, 0.1  $\mu$ g human X (Haematologic Technologies, Inc.); Lane 10 2, 0.1  $\mu$ g human Xa (Haematologic Technologies, Inc.); Lane 3, 0.1  $\mu$ g rX; Lane 4, 0.16  $\mu$ g rX' $\Delta$ 2; Lane 5, 0.13  $\mu$ g rXiN<sub>88</sub>A<sub>185</sub>; Lane 6, 0.15  $\mu$ g rXiA<sub>185</sub>; Lane 7, 0.187  $\mu$ g rX'i( $\Delta$ 2)N<sub>88</sub>, Lane 8, 0.05  $\mu$ g rX'i( $\Delta$ 2)N<sub>88</sub>A<sub>185</sub>.

15           It is evident that, under reducing conditions, human X and human Xa are in dimeric form; human Xa shows a lower molecular weight form of the heavy chain due to the absence of the activation peptide. Recombinant human X in lane 3 is similar to native human X, however some single chain precursor is still evident. In lane 4, 20 recombinant rX' $\Delta$ 2 also shows cleavage to the heavy and light chains. In lanes 5 and 6, the modified recombinant Xi proteins behave in a manner similar to recombinant human X. As expected, lanes 7 and 8 show the presence of monomer, heavy and light chains derived from the 25 proteolytic cleavage of X'i.

#### Example 7

##### Enzymatic Analysis of Recombinant Human Factor X

30           The kinetic measurement of chromozym X (N-methoxycarbonyl-D-norleucyl-glycyl-arginine-4-nitranilide acetate, Boehringer Mannheim) hydrolysis by native human Factor X, Xa, recombinant X (rX), rX' $\Delta$ 0, rX' $\Delta$ 1, rX' $\Delta$ 2, rX' $\Delta$ 3, rXiN<sub>88</sub>A<sub>185</sub>, rXiN<sub>88</sub>, rX'i( $\Delta$ 2)N<sub>88</sub>A<sub>185</sub> and inactivated bovine Xa, Xai-APMSF supplied by Dr. C. 35 Esmon (OMRF, University of Oklahoma) (Skogen, W.F., et

-31-

al., J Biol Chem (1984) 259:2306) were examined at room temperature in 96-well microtiter plates on a Molecular Devices Vmax spectrophotometer. The absorbance at 405 nM was monitored continuously and the reaction velocities were determined directly by the machine and plotted with the Enzfitter program (Elsevier Press). Protein concentrations were determined by ELISA (Example 6). All enzymes were diluted to the appropriate concentrations in 0.1% bovine serum albumin (BSA) 50 mM Tris HCl, pH 8.0, 150 mM NaCl. Duplicate reactions were carried out in 50 mM Tris HCl, pH 8.0, 150 mM NaCl and 2.5 mM  $\text{CaCl}_2$ . All recombinant human Factor X's were Mab717-purified (Example 6) except for  $\text{rX}'\Delta 0$ ,  $\text{rX}'\Delta 1$ , and  $\text{rX}'\Delta 3$  which were purified using QAE-Sepharose (Pharmacia) concentrated (Skogen, W.F., et al., J Biol Chem (1984) 259:2306).

The recombinantly produced peptides derived from vectors containing  $\text{rX}$ ,  $\text{rXiN}_{88}\text{A}_{185}$ ,  $\text{rXiN}_{88}$  and  $\text{rXaiN}_{88}\text{A}_{185}$  were treated by preincubation for 5 minutes with Russell's viper venom to convert them to the Xa or Xai form. Peptides derived from the  $\text{rX}'\Delta 0$ ,  $\text{rX}'\Delta 1$ ,  $\text{rX}'\Delta 2$  and  $\text{rX}'\Delta 3$  vectors were not treated in this fashion.

Figure 7 is a comparison of Lineweaver-Burk plots for native human Factor X and Xa and activated forms derived from recombinant human  $\text{rX}$  and  $\text{rX}'$ . Figure 7a, human X; Figure 7b, human Xa; Figure 7c, human  $\text{rX}$  (treated with Russell's viper venom protease); Figure 7d, human  $\text{rX}'$  (not treated with protease).

Table 2 compares the  $K_{\text{cat}}$  and  $K_{\text{m}}$  values of the recombinantly produced human Factor X's to the native human Factor X and Xa supplied by Haematologic Technologies, Inc.

-32-

Table 2

	Kcat(s <sup>-1</sup> )	Km (μm)	Specificity Constant Kcat/Km (s <sup>-1</sup> M <sup>-1</sup> )
<u>Native forms</u>			
5 X	64	131	489 X 10 <sup>3</sup>
Xa	367	184	1996 X 10 <sup>3</sup>
<u>Precursor construct</u>			
10 rX	22	134	167 X 10 <sup>3</sup>
rX'Δ0	N.D.	-	-
rX'Δ1	N.D.	-	-
rX'Δ2	17	149	115 X 10 <sup>3</sup>
rX'Δ3	N.D.	-	-
15 rXiN <sub>88</sub> AA <sub>185</sub>	N.D.	-	-
rXiA <sub>185</sub>	N.D.	-	-
rX'iN <sub>88</sub> A <sub>185</sub>	N.D.	-	-
rX'iN <sub>88</sub>	N.D.	-	-
Control CHO medium	N.D.	-	-

20

N.D. = not detected, Kcat ≤ .1 in 14 hrs - 16 hrs assay.

Of course, none of the inactivated forms give values; of the rX' forms, only rX'Δ2 showed activity.

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Example 8

Factor X Dependent Prothrombinase Complex Activity  
of Human X, Xa and Recombinant Human rX and rX'

Factor X dependent prothrombinase complex activity was determined by measuring the rate of  
 30 chromozyme TH (tosyl-glycyl-prolyl-arginine-4-nitroanilide acetate, Boehringer Mannheim) hydrolysis by thrombin at room temperature in a 96-well microtiter plate on a Molecular Devices Vmax spectrophotometer. The  
 35 absorbance at 405 nm was continuously monitored and the

-33-

initial one minute reaction velocities were determined directly by the machine and plotted using the Enzfitter program (Elsevier). Reaction mixtures were performed in triplicate with  $0.05 \times 10^{-4} \text{M}$  to  $1.5 \times 10^{-9} \text{M}$  "Factor X,"  
5 determined by ELISA (Example 6),  $0.5 \times 10^{-6} \text{M}$  human prothrombin (STAGO, American Diagnostics, Inc.)  $7.5 \times 10^{-9} \text{M}$  human factor Va (Haematologic Technologies, Inc.),  $20 \times 10^{-6} \text{M}$  phosphocholine/phosphoserine 75%/25% (PCPS) (supplied by Dr. W.R. Church, University of  
10 Vermont), or equivalent amounts of rabbit brain cephalin (Sigma) (Example 9), 0.1% BSA (Sigma),  $0.1 \times 10^{-3} \text{M}$  chromozym TH (Boehringer Mannheim), 25 mM Tris HCl, pH 7.5, 150 mM NaCl and 5 mM  $\text{CaCl}_2$ .

Human Factor rX and rX' dependent  
15 prothrombinase complex activity utilized PCPS and human Factor X and Xa dependent prothrombinase complex activity utilized cephalin. Human Factor X and rX were preincubated for 5 minutes with Russell's viper venom (Haematologic Technologies, Inc.). Thrombin hydrolysis  
20 of chromozym TH as determined by increase of fluorescence signal, was linear throughout the experimental protocol. No observable rates were shown for  $\text{rXiN}_{88}\text{A}_{185}$  at  $59.2 \times 10^{-4} \text{M}$ ,  $\text{rX'iN}_{88}\text{A}_{185}$  at  $10.2 \times 10^{-9} \text{M}$ , or for  $\text{bXai-APMSF}$  at  $1 \times 10^{-9} \text{M}$ . Figures 8a-8d compare Factor X  
25 dependent prothrombinase complex activity of human X (Figure 8a), human Xa (Figure 8b) (Haematologic Technologies, Inc.), recombinant human rX (after treatment with protease) (Figure 8c) and recombinant human  $\text{rX}'\Delta 2$  (after no protease treatment) (Figure 8d).  
30 All are comparably active.

-34-

Example 9Coagulation of Plasma

Mab717 purified rX and rXa were assayed for plasma coagulation activity in an automated two-stage prothrombin assay on a MLA Electra 800 fibrometer. Enzyme protein concentrations were determined by ELISA (Example 6) and diluted in 0.1% BSA, 150 mM NaCl prior to use. Bovine Factor X and Factor VII deficient plasma (Sigma) and rabbit brain cephalin (Sigma) were prepared according to manufacturers' instructions. Russell's viper venom 0.1 µg/ml was added to human X and rX assays. The reaction mixture comprised 0.1 ml Factor X, 0.1 ml 150 mM NaCl, 0.1 ml cephalin and 0.1 ml 25 mM CaCl<sub>2</sub>. Duplicates were performed on each concentration and the average of two experiments were calculated. Figure 9 compares the plasma coagulation activity of human X, human Xa, human rX and human rXa. Human rX was calculated to be 45% as active as human X and human rXa was calculated to be 32% as active as human Xa.

Example 10Inhibition of ProthrombinaseComplex Activity by rXiN88A185.Human rX'i(Δ2)N88A185 and Bovine bXai-APMSF

Inhibition of native human Factor X dependent prothrombinase complex activity by human rXiN<sub>88</sub>A<sub>185</sub> and inhibition of native human Factor 5 x 10<sup>-9</sup>M Xa dependent prothrombinase complex activity by human rX'i(Δ2)N<sub>88</sub>A<sub>185</sub>(rXai) and bovine bXai-APMSF (C. Esmon, OMRF, University of Oklahoma) was tested as detailed in Example 8. It is necessary to compare directly X with Xi and Xa with Xai because of kinetic factors and the strength of the complex once formed. Human rXiN<sub>88</sub>A<sub>815</sub> was preincubated for 5 minutes with 0.1 µg/ml Russell's

-35-

viper venom. The human Factor X and Xa concentrations were  $1 \times 10^{-9}$  M.

Figure 10 shows the concentration dependent inhibition of the human Factor Xa dependent prothrombinase complex by bXai-APMSF, rX'i( $\Delta 2$ )N<sub>88</sub>A<sub>185</sub>(rXai) and inhibition of the human Factor X dependent prothrombinase complex by rXiN<sub>88</sub>A<sub>185</sub>. 50% inhibition by bXai-APMSF was obtained at  $0.9 \times 10^{-9}$  M, 50% inhibition by rX'i( $\Delta 2$ )N<sub>88</sub>A<sub>185</sub> was obtained at  $6 \times 10^{-9}$  M and 50% inhibition by rXiN<sub>88</sub>A<sub>185</sub> was obtained at  $10.6 \times 10^{-9}$  M.

#### Example 11

##### Preparation of Acylated Factor Xa

Human recombinant Xa (rXa) is prepared as described in Example 5 and cleaved with Russell's Viper Venom. The rXa is dissolved in saline solution ( $2 \mu\text{mol}/25 \text{ ml}$ ) and treated with  $5 \mu\text{mol}$  of the para-nitrophenyl ester of p-toluoylic acid with stirring for 20 minutes at  $4^\circ\text{C}$ . The resulting reaction mixture is dialyzed against glycerol/saline and the dialysate is stored at  $0^\circ\text{C}$ .

-36-

CLAIMS

1. A two-chain Factor Xai peptide having at least 80% homology with the amino acid sequence of light chain positions 1-139 and heavy chain positions 1-254 of Figure 1 wherein said Factor Xai is capable of competing with Factor Xa in the formation of a prothrombinase complex and wherein said Factor Xai does not result in proteolytic activity when included in said complex.
2. The Factor Xai of claim 1 wherein the serine residue corresponding to that at position 185 of the heavy chain in Figure 1 is replaced by a different amino acid and/or the aspartic acid residue at the position corresponding to that of position 88 of the heavy chain shown in Figure 1 is replaced by an alternate amino acid.
3. The Factor Xai of claim 2 wherein the replacement for serine is an alanyl residue and the replacement for aspartic acid is an asparagine residue.
4. A single-chain precursor polypeptide which is convertible to Factor Xai by proteolysis; said Factor Xai having at least 80% homology with the amino acid sequence of light chain positions 1-139 and heavy chain positions 1-254 of Figure 1 wherein said Factor Xai is capable of competing with Factor Xa in the formation of a prothrombinase complex and wherein said Factor Xai does not result in proteolytic activity when included in said complex.
5. The polypeptide precursor of claim 4 which is reactive with antibodies raised against Factor X.



-37-

6. The polypeptide precursor of claim 4 wherein the serine residue corresponding to that at position 185 of the heavy chain in Figure 1 is replaced by an alternate amino acid and/or the aspartic acid residue at the position corresponding to that of position 88 of the heavy chain shown in Figure 1 is replaced by an alternate amino acid.

7. The polypeptide precursor of claim 6 which, in unmodified form comprises an amino acid sequence of amino acids 1-139 of the light chain and amino acids 1-254 of the heavy chain shown in Figure 1.

8. The polypeptide precursor of claim 6 wherein said light chain and heavy chain are linked by an amino acid sequence at least 80% homologous to the activation peptide of Figure 1.

9. The polypeptide of claim 6 wherein said light chain and heavy chain are linked by an amino acid sequence consisting essentially of a protease cleavage site.

10. The polypeptide of claim 9 wherein said cleavage site is RKRRKR wherein said light chain and heavy chain are linked by an amino acid sequence consisting essentially of a protease cleavage site.

11. A DNA sequence which encodes the precursor polypeptide of claim 4.

-38-

12. A recombinant expression vector capable of expressing a DNA sequence encoding the precursor polypeptide of claim 4 when said expression system is transfected transformed into host cells and said host cells are cultured under conditions favorable to said expression, which expression system comprises a DNA sequence encoding the precursor polypeptide of claim 4 operably linked to control sequences for the expression of said DNA.

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13. Recombinant host cells transformed with the expression system of claim 12.

14. A method to produce single-chain precursor polypeptide which is convertible by proteolysis to Factor Xai,

which method comprises culturing the cells of claim 13 under conditions favorable for the expression of the DNA encoding said precursor polypeptide; and

recovering the precursor polypeptide produced or the proteolysis products thereof.

15. A pharmaceutical composition for the prevention or treatment of thrombosis in animal subject, which composition comprises an amount of the Factor Xai of claim 1 effective to ameliorate or prevent thrombosis in admixture with a pharmaceutically acceptable excipient.

16. A pharmaceutical composition for the prevention or treatment of inflammation in animal subject, which composition comprises an amount of the Factor Xai of claim 1 effective to ameliorate or prevent inflammation in admixture with a pharmaceutically acceptable excipient.

-39-

17. A pharmaceutical composition for the prevention or treatment of restenosis in animal subject, which composition comprises an amount of the Factor Xai of claim 1 effective to ameliorate or prevent restenosis in admixture with a pharmaceutically acceptable excipient.

18. A pharmaceutical composition for the prevention or treatment of complications of transplantation in animal subject, which composition comprises an amount of the Factor Xai of claim 1 effective to ameliorate or prevent complications of transplantation in admixture with a pharmaceutically acceptable excipient.

19. A method to prepare Factor Xai useful in treatment of thrombosis, which method comprises contacting the precursor polypeptide of claim 4 with an amount of a protease effective to cleave said precursor; and recovering the Factor Xai produced.

20. The method of claim 19 wherein said protease is contained in a Russell viper venom extract.

21. A Factor Xai prepared by the method of claim 19.

22. Factor Xa which has been modified to extend its half-life in serum wherein said modified form of factor Xa generates active factor Xa in the presence of serum.

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-40-

23. The modified factor Xa of claim 22 which is acylated Factor Xa.

24. A pharmaceutical composition for use in treating hemophilia which comprises the modified Factor Xa of claim 22 in admixture with a pharmaceutically acceptable excipient.

25. A method to prevent or treat thrombosis in an animal subject which method comprises administering to a subject in need of such treatment an effective amount of the Factor Xai of claim 1 or a pharmaceutical composition thereof.

26. A method to prevent or treat inflammation in an animal subject which method comprises administering to a subject in need of such treatment an effective amount of the Factor Xai of claim 1 or a pharmaceutical composition thereof.

27. A method to prevent or treat restenosis in an animal subject which method comprises administering to a subject in need of such treatment an effective amount of the Factor Xai of claim 1 or a pharmaceutical composition thereof.

28. A method to prevent or treat complications of transplantation in an animal subject which method comprises administering to a subject in need of such treatment an effective amount of the Factor Xai of claim 1 or a pharmaceutical composition thereof.

29. A method to treat hemophilia in human subjects which method comprises administering to a subject in need of such treatment an amount of Factor Xa

-41-

which has been modified to extend its half-life in serum effective to offset the effects of hemophilia in said subject.

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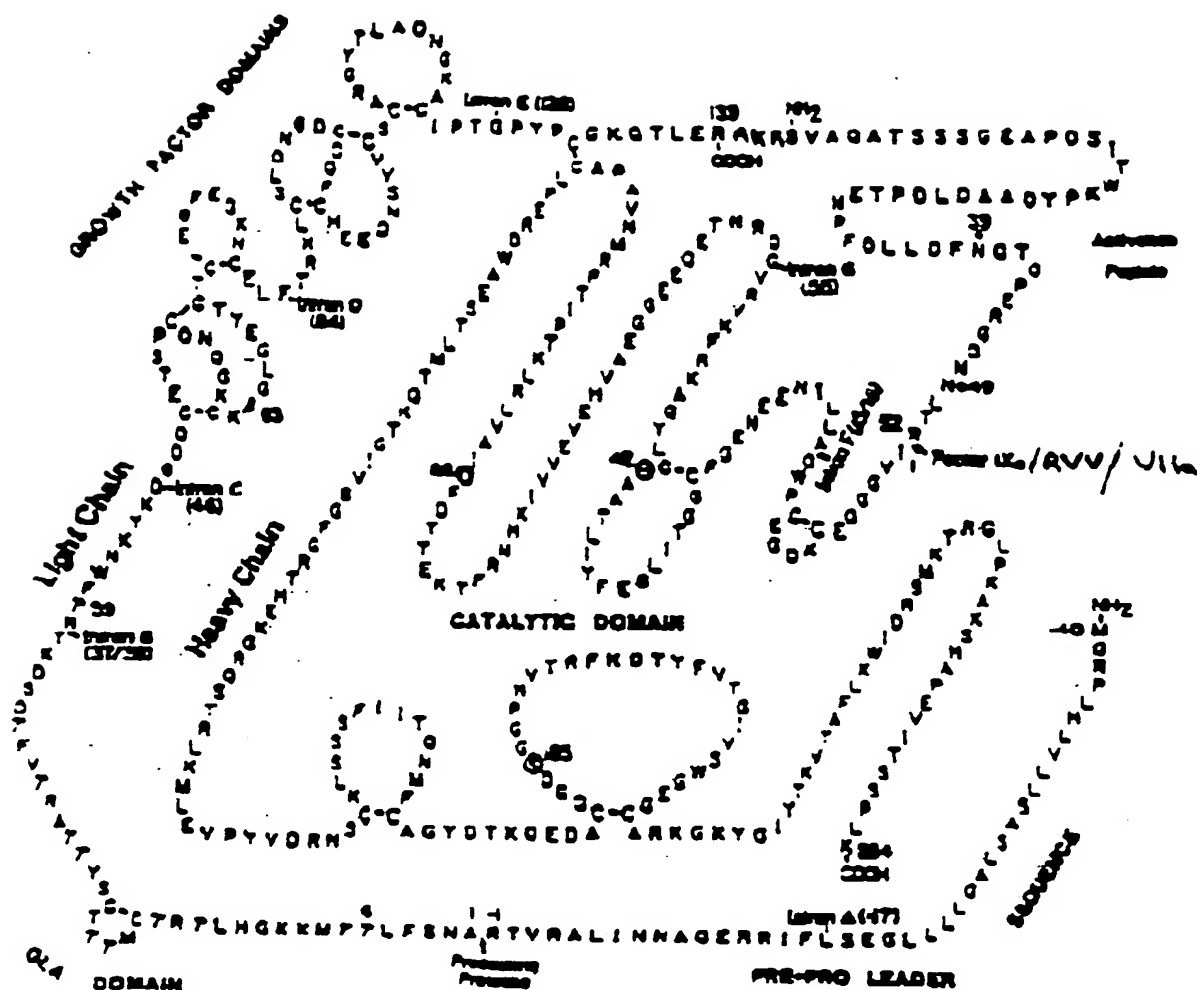


Figure 1. Human Factor X.

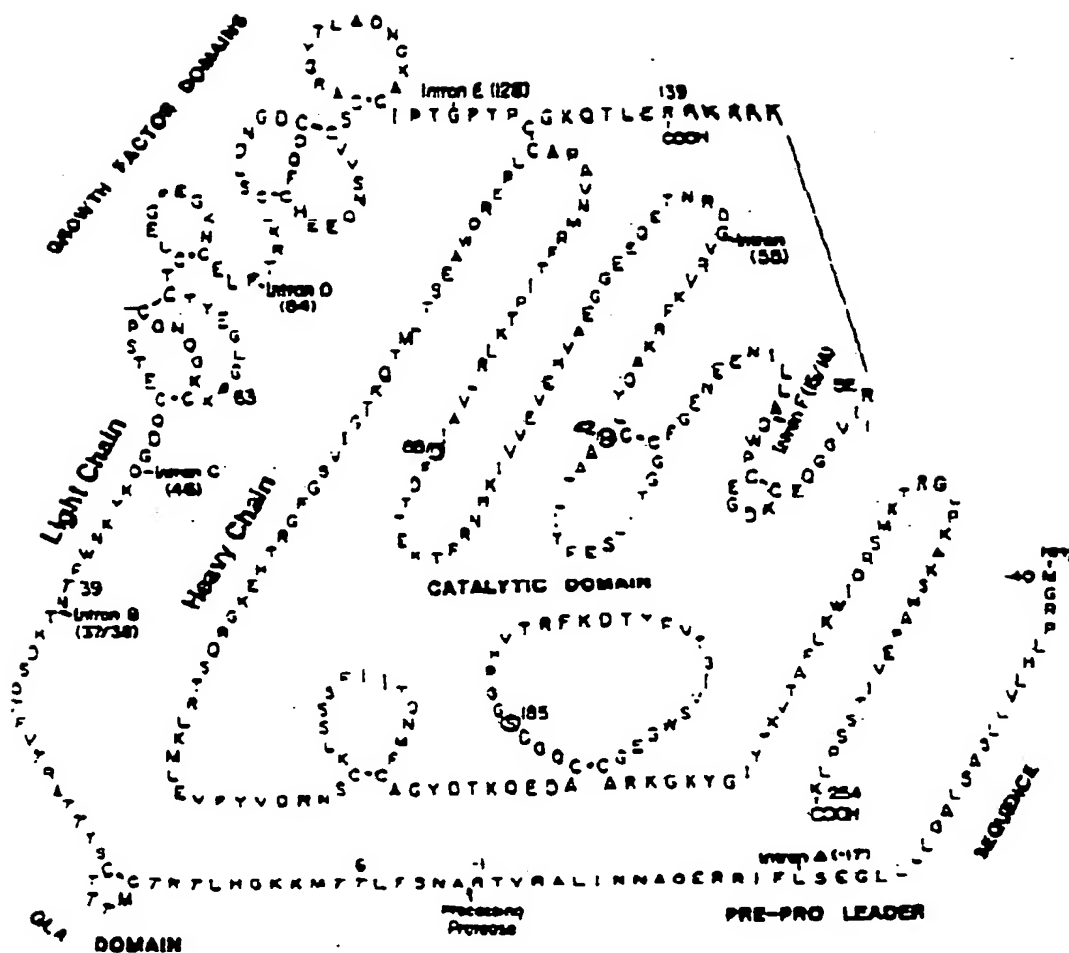


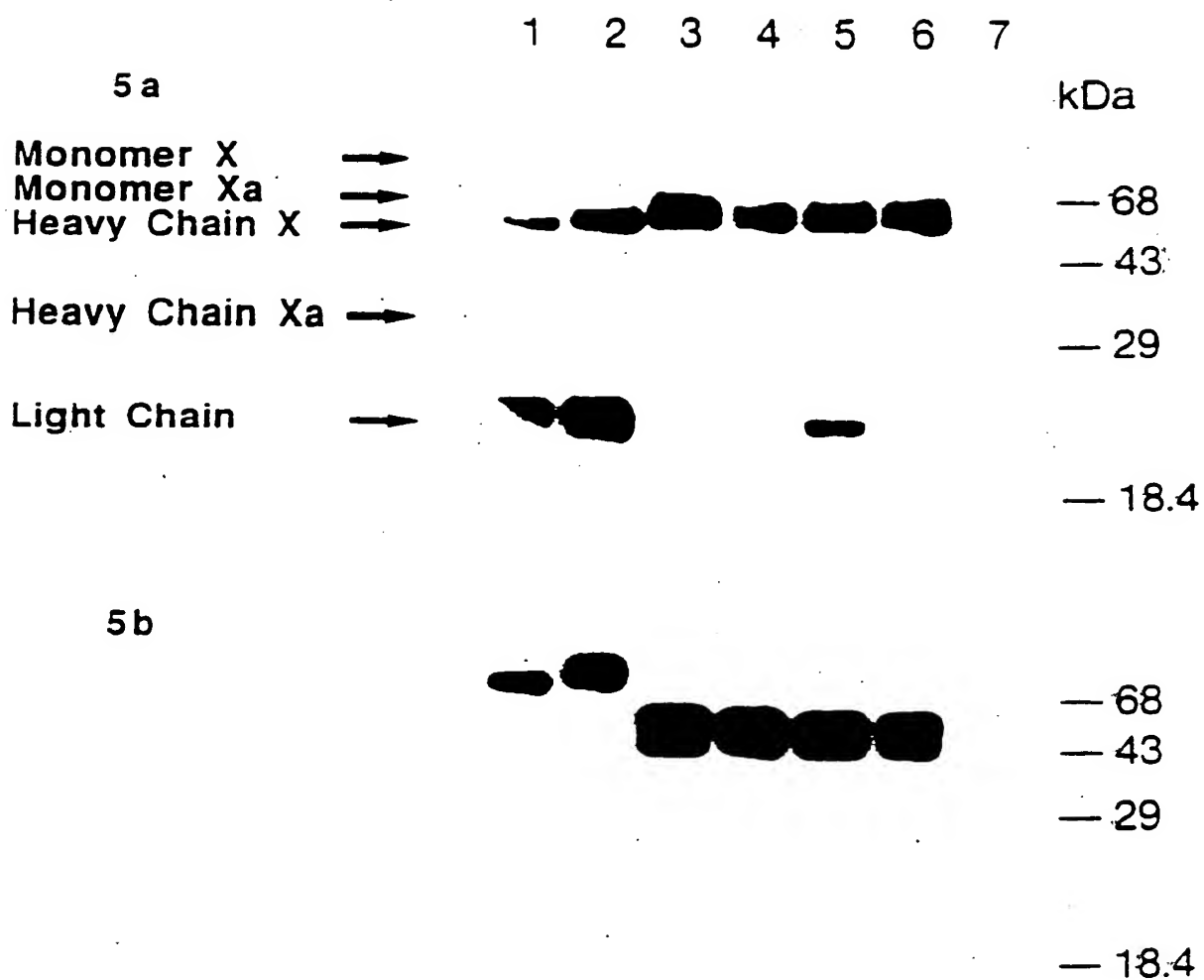
Figure 2. Recombinant human Factor X'(rX').

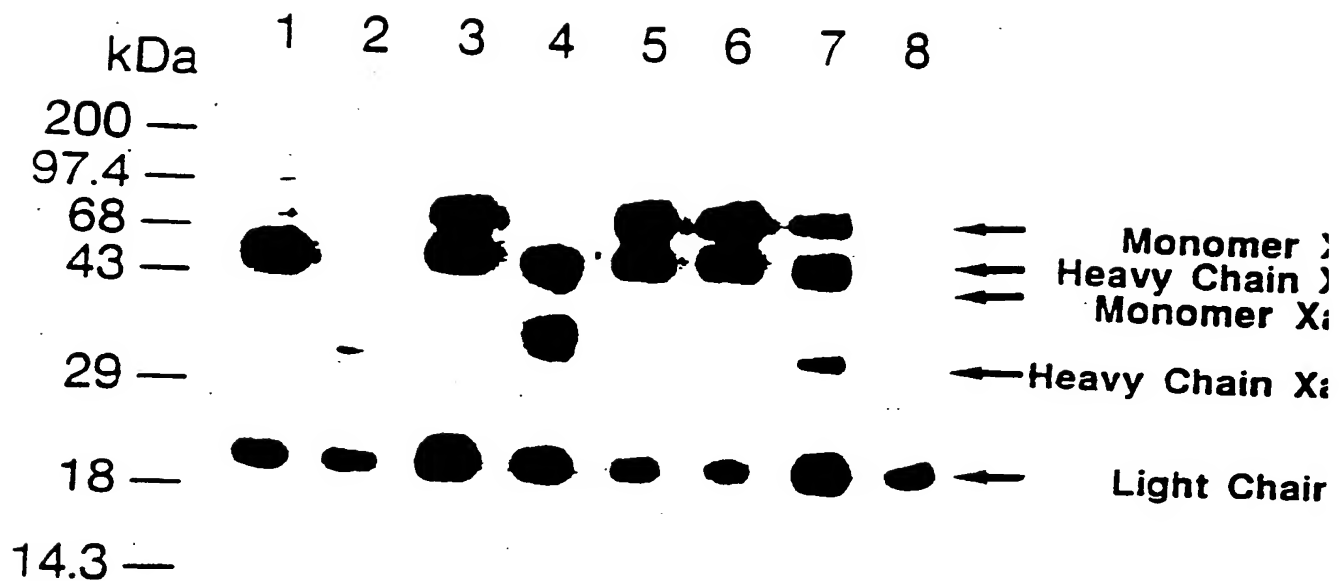




1 STGACTGTA GA AGCTGG CAGGAATTC CGATGCGCGG CCGACT AG  
21 CTGCTGCTGG TCAGTGGCTC CTTGGGCTGG CTGCTGCTGC TTTGGGAAAG  
31 TTTGTCATC CCGAGGGAGC AGGCAACAA CATCTGCGG AGGGTCACGA  
41 CCGCCGATTC CTTCTTTGAA GACATGAAGA AACGACACCT CCAAAGAGAG  
51 TCATGGGAAG AGAGCTGCTC ATACGAAGAG GCGCGCGAGG TTTTGGAGGA  
61 CAGCGACAAG ACGAATGAAT TGTGGAATAA ATACAAAGAT GCGGACCACT  
71 GTGACACCAG TCGTGGCAG AACGAGGCA AATGTAAAGA CCGGCTCGCG  
81 GAATACACCT GCAGCTGTTT AGAAGGATTC GAAGCCAAAA ACTGTGAATT  
91 ATTACACCGG AAGCTGTGCA GCCTGGACAA CCGGGACTGT CACCACTTCT  
101 GCGACGAGGA ACAGAACTGT GTGTTGTGCT CTTGGGCGCG CCGGTACACC  
111 CTGGTGACA ACCGCAAGGC CTGCATTGCG ACAGGGGCGT ACCCTGTGG  
121 GAAACAGACC CTGGAACGCA GGAAGAGGTC AGTGGGCGAG GCGACGAGCA  
131 GCAGCGGGGA GCGCGCTGAC AGCATCACAT GGAAGCCATA TGAATCAGCG  
141 CAGCTGGAGC CCACCGAGAA CCGCTTGAC CTGCTTCACT TCAACGAGC  
151 GCAGCGTGAC AGGGGCGACA ACAACCTCAC CAGGATCGTG CGAGCGGAGG  
161 AATCCAAGCA CCGGGAGTGT CCGTGGCAGG CCGTGTCTAT CAATGAGGAA  
171 AACGAGGGGT TGTGTGCTGG AACATCTG AGCGAGTCT ACATGCTAAG  
181 GGCAGGGCAG TGTCTCTACC AAGCGAAGAG ATTG AGGTTG AGGTA AGGG  
191 ACCGGAACAC GAGGAGGAG GAGGGCGGTG AGGCGGTGCA CGAGGTGGAG  
201 GTGTCATCA AGCACAACCG GTTCACAAAG GAGACCTATG ACTTCGACAT  
211 CCGCTGCTC CCGGTCAAGA CCGCATCAC CTTCGCGATG AACGTGCGG  
221 CTGCTGCTGT CCGCGAGGCT GACTGGGCG AGTCCAGGCT GATGAGGCAG  
231 AAGACGGGGA TTGTGAGCGG CTTCGGGCGC ACCCAGGAGA ACGGCGGCGA  
241 GTCCACGAGG CTCAGATGC TCGAGGTGCC CTAGCTGGAC CGCAACAGCT  
251 CCAAGCTGTC CAGCAGCTTC ATCATCACC AGAAGATGTT CTGTGCGCGG  
261 CGTACCGCGC TTCAAACACA CCGACTTCTT GACAGGCATC GTTACGCTGG  
271 CAGAGCGGTC TCGCGTAAG GCGAAGTACC CGATCTACAC CAAGTCCAGC  
281 GCGTCTCTCA AGTGGATCGA CAGTCCATC AAAACCAAGC GGTTCGCGAA  
291 GCGCAAGAGC CATGCGCGCG AGGTCAATAAG CTCTGTGCA TTAACTGAG  
301 ATCCCACTCA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA

FIGURE 4

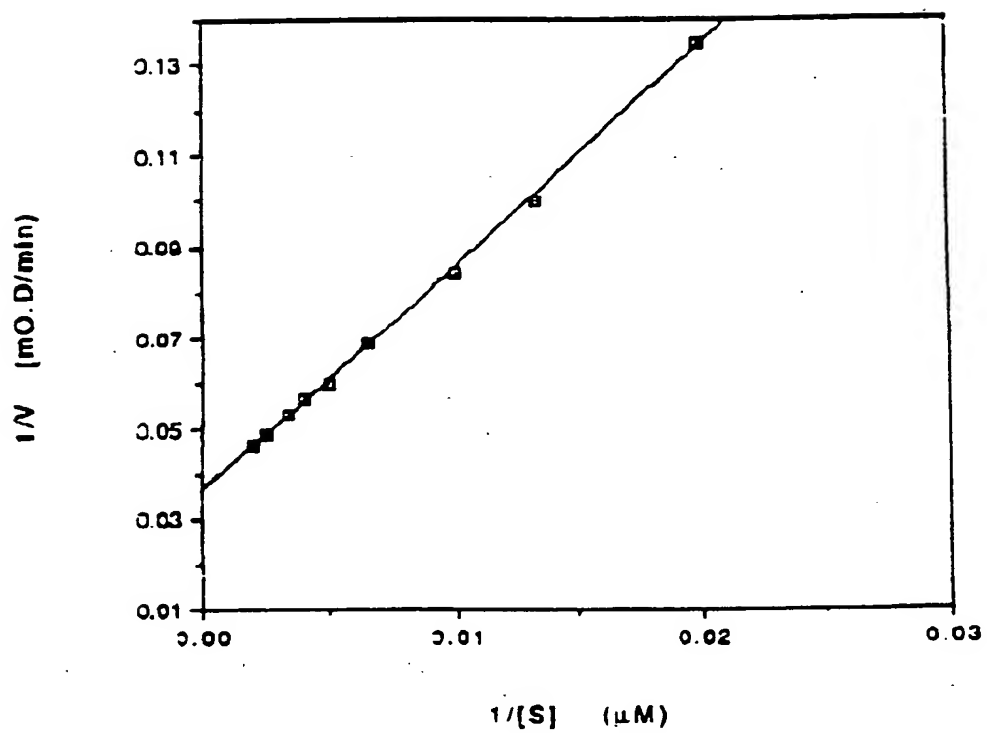
**FIGURE 5a, 5b****FIGURE 5. Western Blot Analysis of Recombinant Human Factor X's**

**FIGURE 6**

**FIGURE 6.** Western Blot Analysis of Recombinant Human Factor X's

7 / 16

Figure 7a. Lineweaver-Burk plot of X.



8 / 16

Figure 7b. Lineweaver-Burk plot of Xa.

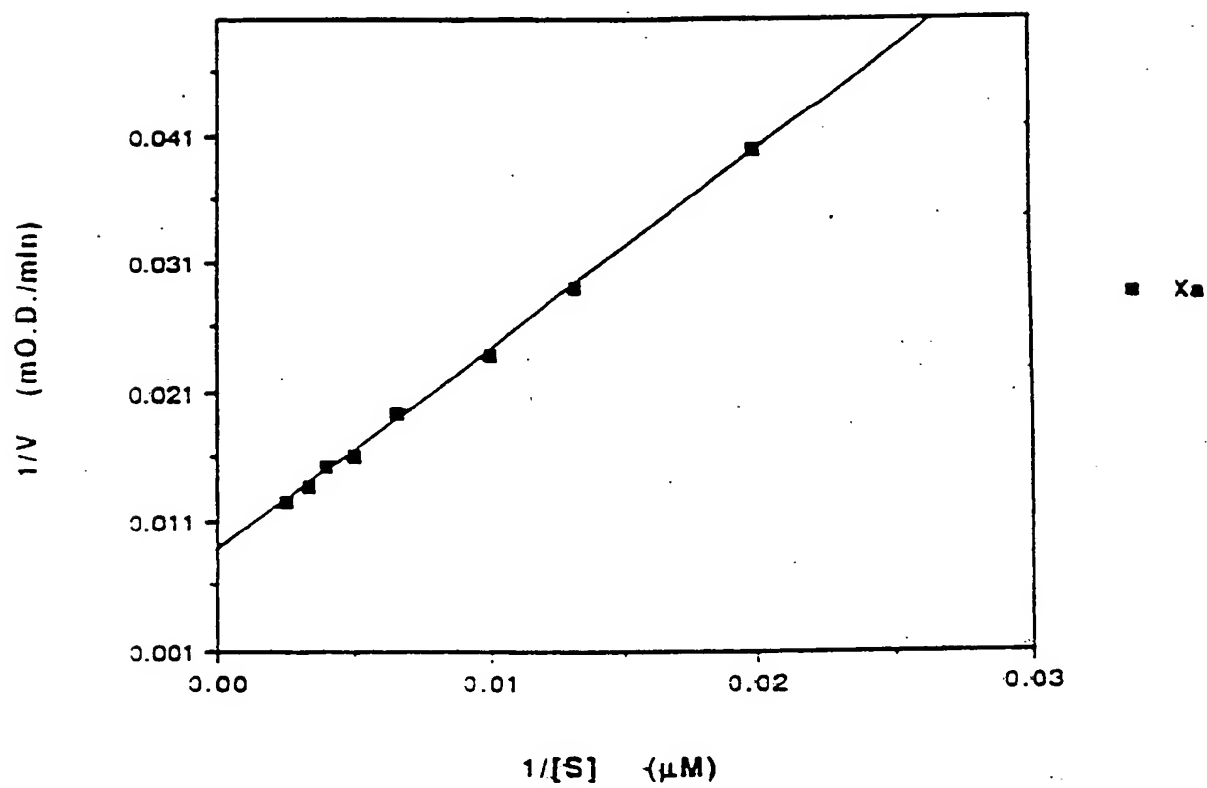
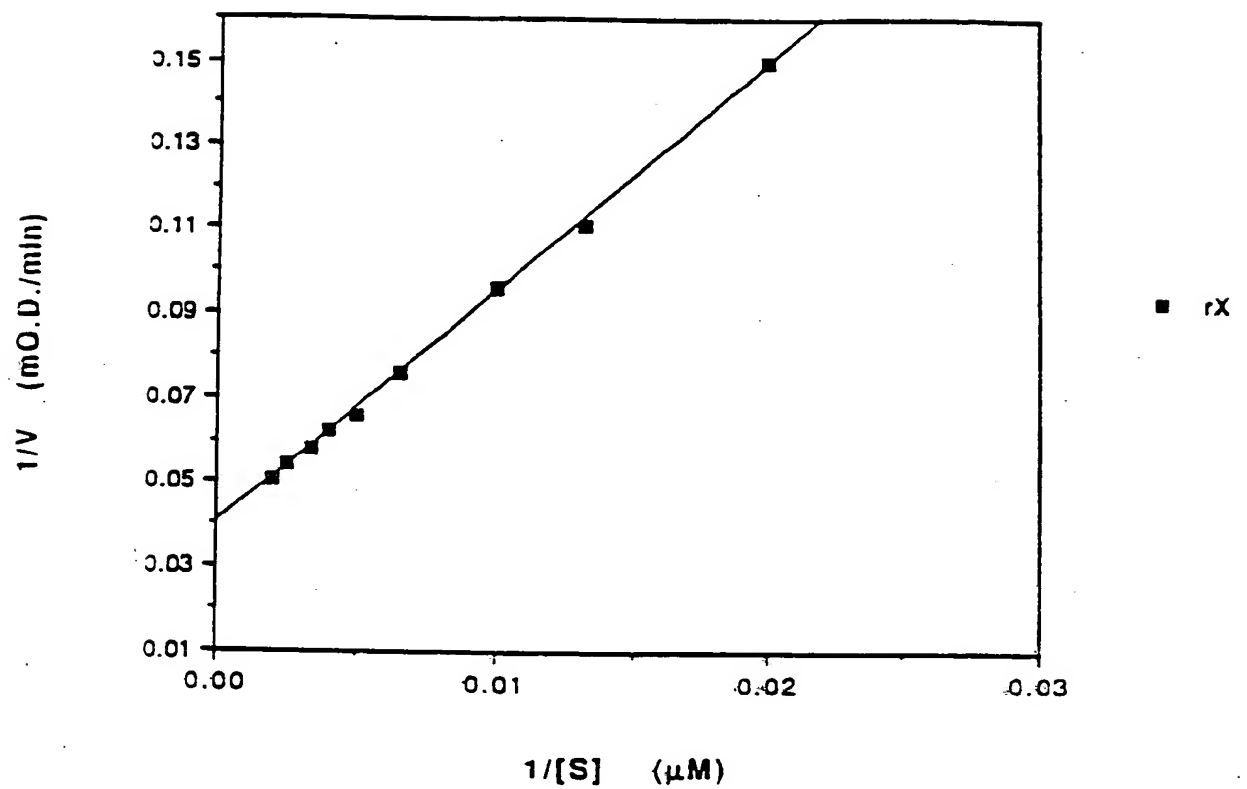
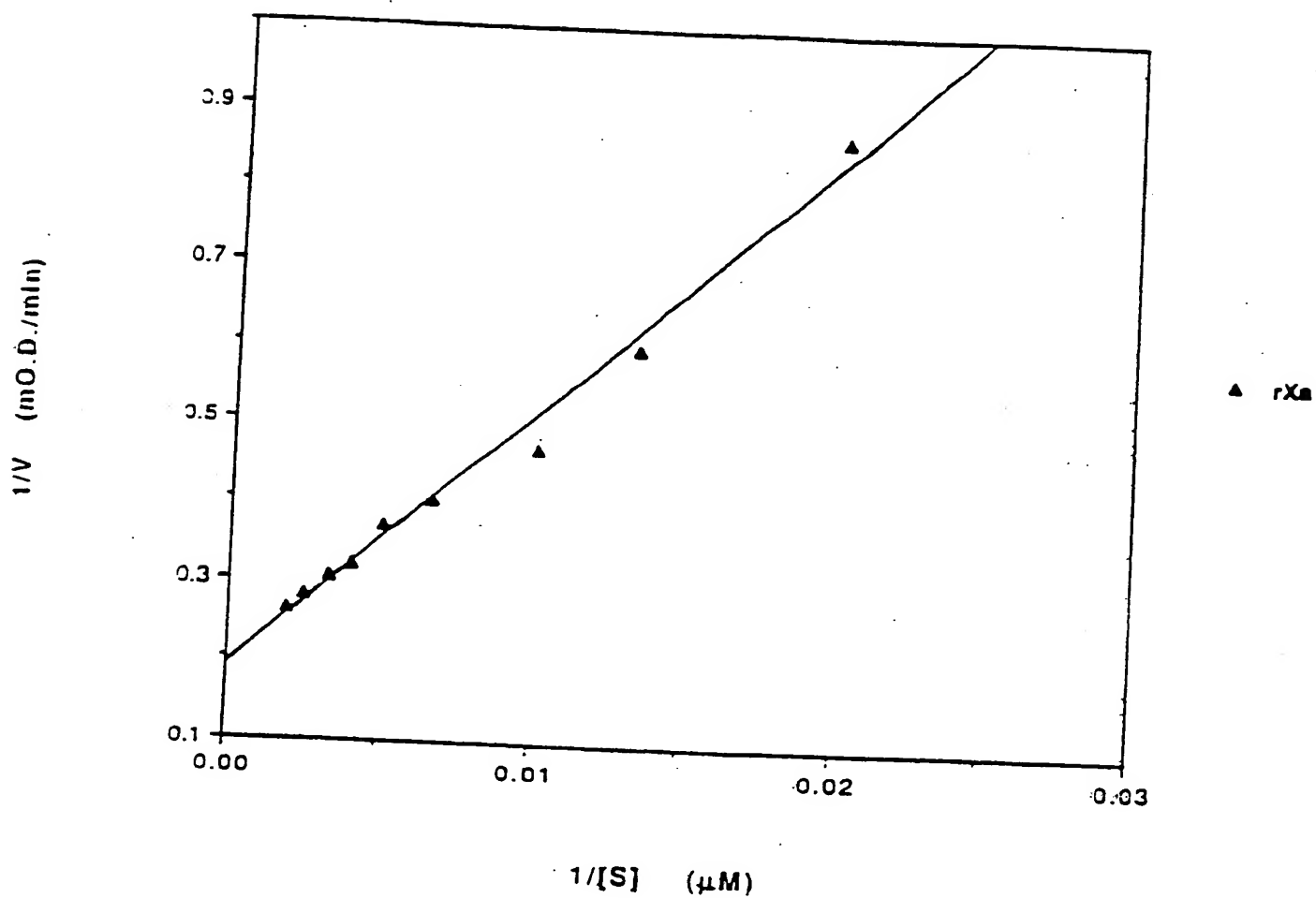


Figure 7c. Lineweaver-Burk plot of rX.



10/16

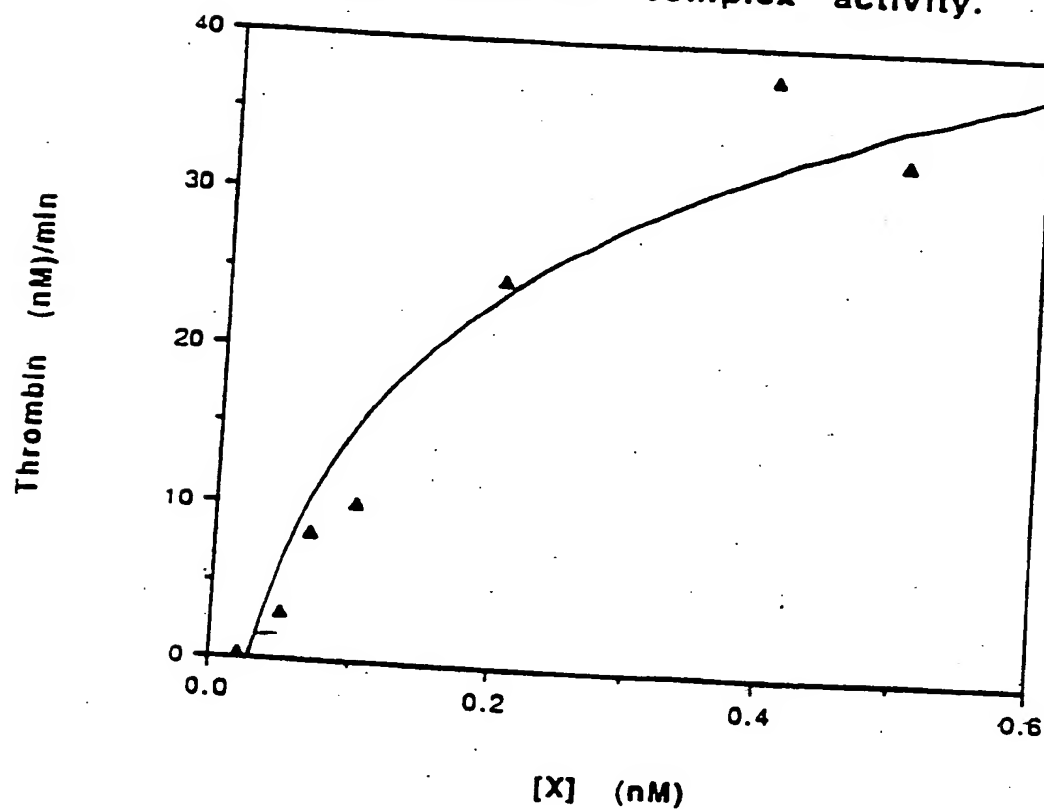
Figure 7d. Lineweaver-Burk plot of rXa.



11/16

FIGURE 8a

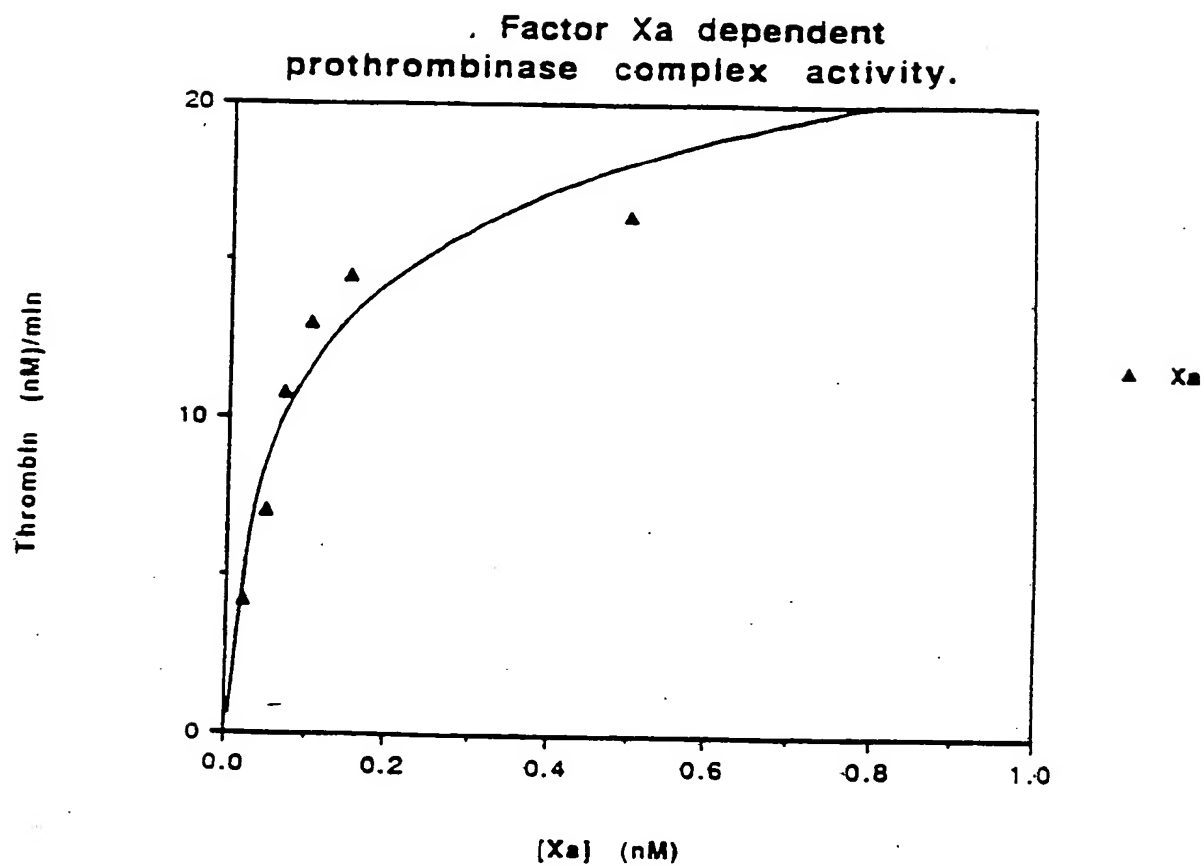
Factor X dependent  
prothrombinase complex activity.





12/16

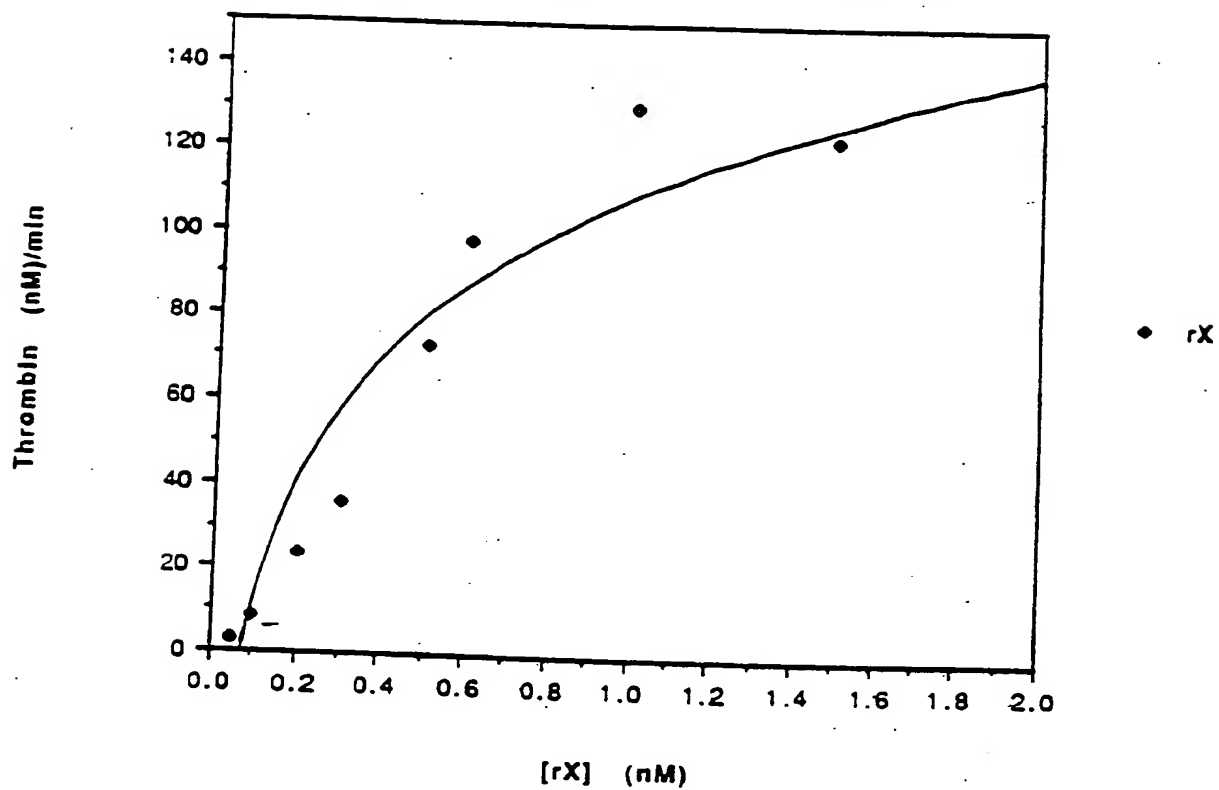
FIGURE 8b



13/16

FIGURE 8c

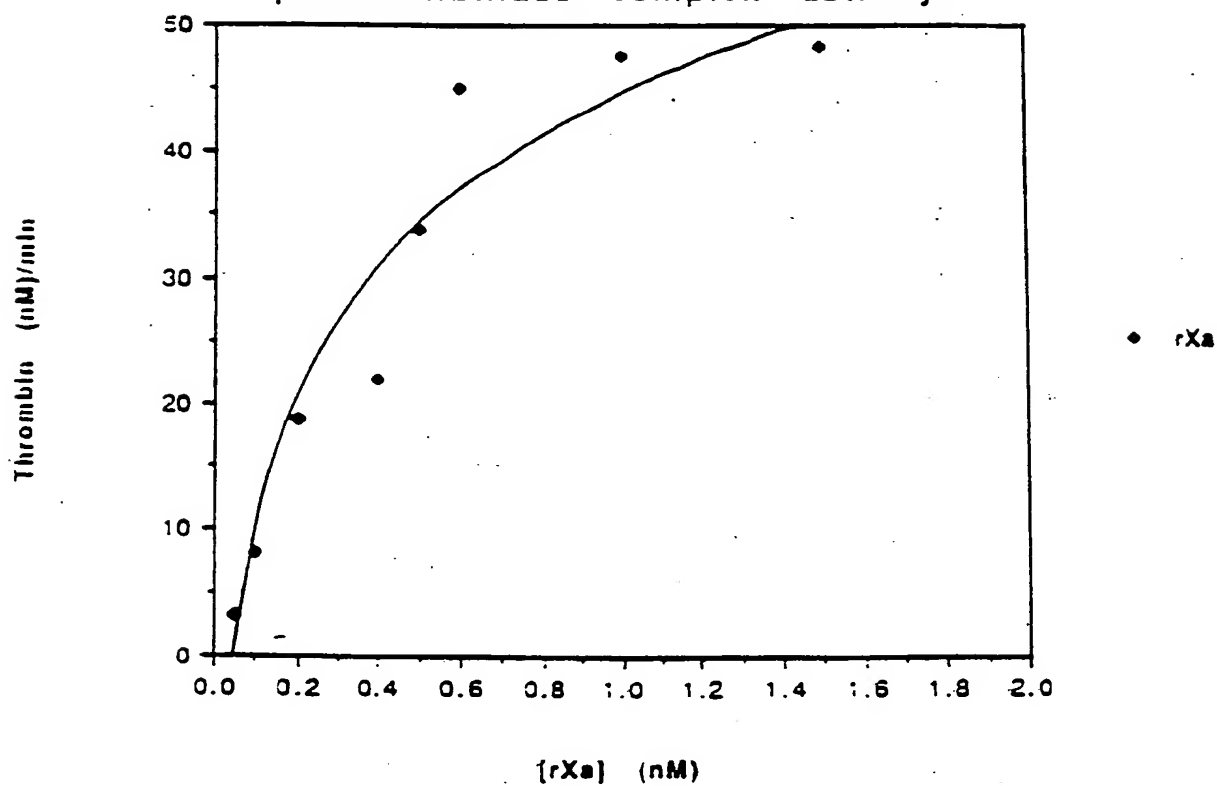
Factor rX dependent  
prothrombinase complex activity.



14 / 16

FIGURE 8d

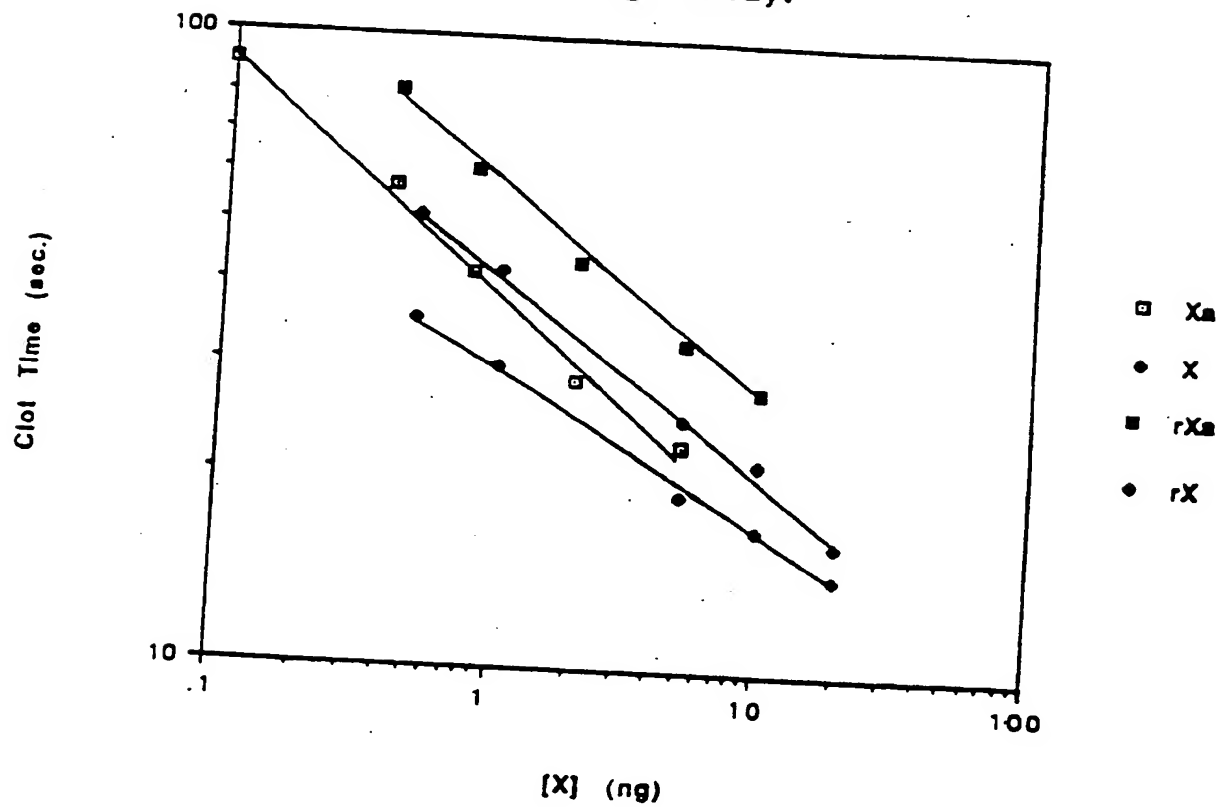
Factor rX' dependent  
prothrombinase complex activity.



15 / 16

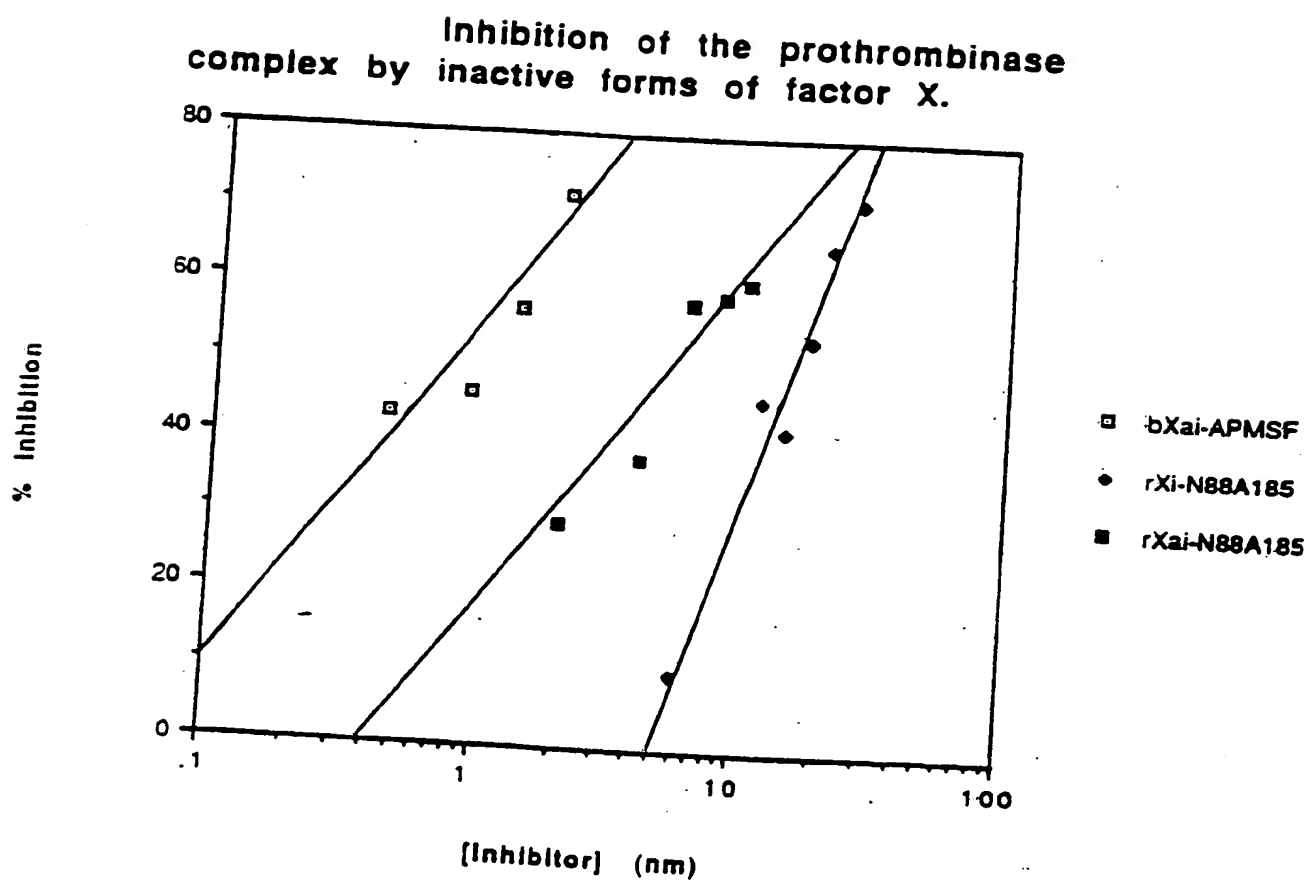
FIGURE 9

Factor X dependent two stage  
prothrombin clotting assay.



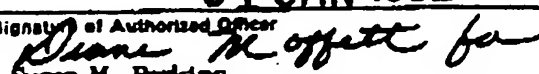
16 / 16

FIGURE 10



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06337

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>1</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C07K 13/00; A61K 37/02, 35/14; C07H 15/12, 17/00; C12P 21/06 U.S. Cl.: 514/8, 12; 530/384, 395, 435/69.1, 69.2, 69.6, 71.1; 536/27		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>2</sup>		
Classification System	Classification Symbols	
U.S. Cl.	514/8, 12; 530/384, 395; 435/69.1, 69.2, 69.6, 71.1; 536/27	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>3</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>4</sup></b>		
Category <sup>5</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	Journal of Biological Chemistry, Volume 262, No. 27, issued 25 September 1987, E.J. Husten et al., "The Active Site of Blood Coagulation Factor Xa", pages 12953-12961.	1-29
A	Journal of Biological Chemistry, Volume 256, No. 13, issued 10 July 1981, M.E. Nesheim et al., "Cofactor Dependence of Factor Xa Incorporation into the Prothrombin Complex", pages 6537-6540.	1-29
A	Journal of Biological Chemistry, Volume 259, No. 4, issued 25 February 1984, N. F. Skogen et al., "Comparison of Coagulation Factor Xa and Des-(1-44) factor Xa in the Assembly of Prothrombinase", pages 2306-2310.	1-29
A	Journal of Biological Chemistry, Volume 263, No. 8, issued 15 March 1988, S. Krishnasamy, "Prothrombinase Complex Assembly", pages 3823-3834.	1-29
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<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
23 January 1991		31 JAN 1992
International Searching Authority		Signature of Authorized Officer
ISA/US		 Susan M. Perkins

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